



Novel Regulators of Liver Development and Metabolism

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Novel regulators of liver development and metabolism

A dissertation presented

by

Leah Yu Liu

to

The Division of Medical Sciences

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

in the subject of

Biological and Biomedical Sciences

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Novel regulators of liver development and metabolism

Abstract

Chronic liver diseases such as non-alcoholic fatty liver disease and alcoholic liver disease are significant health concerns worldwide. Despite knowledge of disease features and environmental causes, we lack understanding of the genetic factors and molecular mechanisms that can be targeted for liver disease therapeutics. Many of these factors are also essential during embryonic development and organogenesis. Here, we use liver development in zebrafish as a model and paradigm for the discovery of regulators impacting liver disease pathogenesis. A chemical screen in zebrafish identified the endocannabinoid (EC) signaling pathway as a regulator of liver development. This pathway was previously implicated in animal models of chronic liver disease, but little was known about its role in development. We generated cannabinoid receptor mutant zebrafish using genome editing and show that EC signaling is required for hepatic maturation and outgrowth, but not earlier milestones such as hepatic specification. Mutant zebrafish also exhibited defects in lipid processing, and we found that methionine metabolism, involving sterol regulatory element binding proteins (SREBPs), is an integral mediator in this process. In a separate study, we used zebrafish to functionally annotate a panel of candidate genes identified by a genome-wide association study (GWAS) for elevated liver plasma enzymes, which are used as a clinical liver disease marker. We prioritized GWAS candidates for morpholino knockdown in zebrafish and discovered effects on hepatic progenitor and hepatocyte development as well as differences in susceptibility to metabolic and toxic injury. Our approach can be applied to other GWAS data sets to rapidly assess additional characteristics

during zebrafish development. The work presented here gives valuable insight into how regulators of hepatogenesis also have roles in metabolism and disease.

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Chapter 1: Introduction

Chronic liver disease

Chronic liver diseases such as non-alcoholic fatty liver disease and alcoholic liver disease (NAFLD and ALD, respectively) contribute to significant morbidity and mortality around the world. NAFLD currently affects up to 38% of the U.S. population (Anstee and Day, 2013) and 20-30% of European, Middle Eastern, and East Asian populations. Prevalence of NAFLD is projected to increase as more people worldwide adopt a Western-style diet and lifestyle, which contributes to obesity (Loomba and Sanyal, 2013). ALD is estimated to account for 10% of disability-adjusted life-years in developed countries, causes a greater number of liver disease deaths in the US than hepatitis C, and is second only to viral hepatitis as the major cause of liver transplantation (Altamirano and Bataller, 2011). ALD and NAFLD follow a similar disease progression despite their different causes: early stages of chronic liver disease include hepatic steatosis or fatty accumulation in the liver, followed by hepatitis or inflammation. Prolonged exposure leads to liver cell death and scar tissue formation called cirrhosis, which is irreversible, and these disease states predispose to cancers such as hepatocellular carcinoma (HCC). Liver transplantation can be curative but organs are scarce and patients are required to undergo lifelong immunosuppression. Unfortunately, no effective drug options for chronic liver disease currently exist, and early diagnosis is difficult due to lack of prognostic symptoms and a limited understanding of disease mechanisms. The goal of our lab and others is to characterize the molecular mechanisms of liver disease pathogenesis to reveal processes that can be targeted for novel therapeutics.

The liver is the largest human internal organ and its essential metabolic functions include maintenance of glucose homeostasis, detoxification of drugs, and cholesterol/fat synthesis, which all exhibit compromised function during disease. The liver also produces essential factors, such

as bile to digest lipids and coagulation proteins to clot blood (Si-Tayeb et al., 2010; Zorn, 2008). Parenchymal cells called hepatocytes, which comprise approximately 70-80% of the liver, perform most of these functions (Figure 1-1). Mammalian liver cellular architecture consists of hepatocytes arranged in a hexagonal lobule with blood-filled sinusoids surrounded by the “portal triad.” The portal triad consists of the bile duct, portal vein, and hepatic artery that supply blood to the liver, with blood exiting through the central vein at center of the lobule (Si-Tayeb et al., 2010). Secretion of bile occurs from the apical face of the hepatocytes adjacent to bile canaliculi and into the intrahepatic and extrahepatic bile ducts and gallbladder (Zorn, 2008). Non-parenchymal liver cells include the Kupffer cells, which are liver-resident macrophages, and the hepatic stellate cells (HSC), which are perisinusoidal cells that mediate liver fibrosis. In response to liver injury, HSCs transdifferentiate from vitamin A storage cells into fibrogenic myofibroblast cells that secrete cytokines, reactive oxygen species (ROS), and inflammatory mediators. In adult mammals, the liver can regenerate up to 70% of its mass, primarily through proliferation of differentiated hepatocytes (Si-Tayeb et al., 2010).

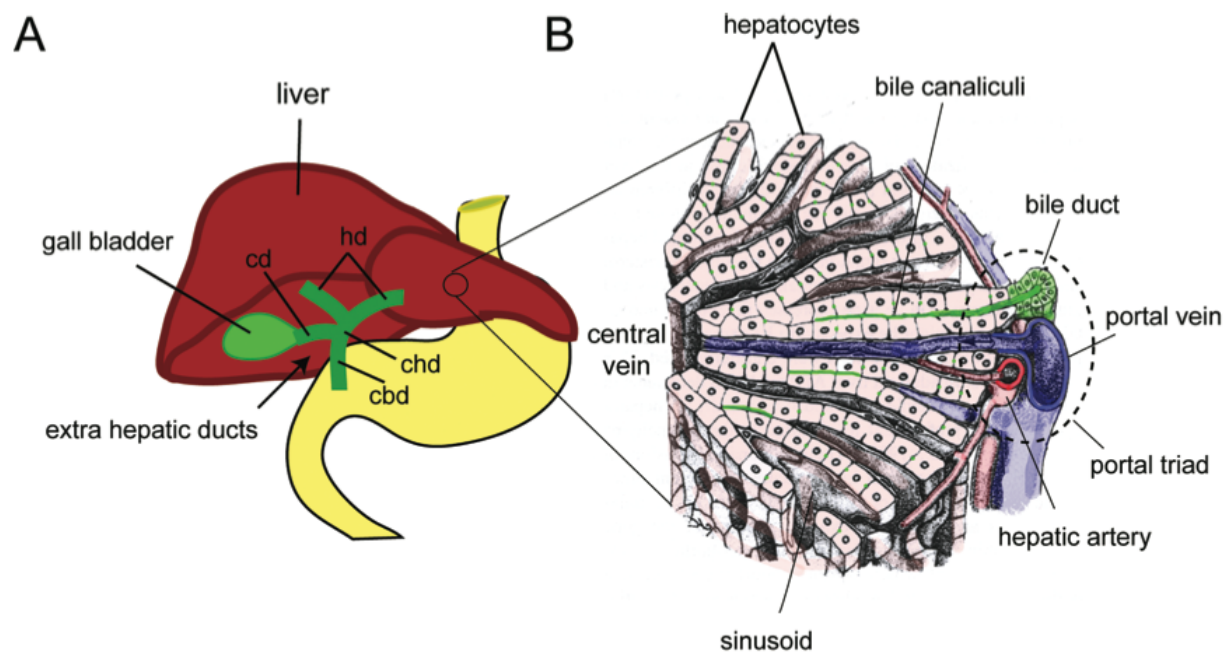


Figure 1-1. Mammalian liver morphology and cellular architecture (Zorn, 2008)

A) Digestive organ morphology including the liver (red), gall bladder and bile ducts (green), and stomach (yellow).

B) Liver cellular architecture showing hepatocytes emanating from a central vein and flanked by the portal triad of a bile duct, portal vein, and hepatic artery.

Hepatic steatosis, also known as fatty liver, refers to lipid accumulation inside hepatocytes. Although steatosis by itself is reversible, continued fatty deposition leads to inflammation and irreversible fibrosis, which alters liver structure and function (Figure 1-2). Steatosis is a defining feature of both ALD and NAFLD. NAFLD is the liver manifestation of obesity and the metabolic syndrome, which is strongly correlated with features such as insulin resistance, glucose intolerance, and type 2 diabetes (Anstee and Day, 2013). As obesity continues to impact a growing proportion of the population, NAFLD rates have increased, with 67% of overweight and 94% of obese individuals affected (Anstee and Day, 2013). ALD patients also have steatosis, which is instead triggered by excess alcohol consumption that initiates pro-lipogenic pathways in the liver. ALD patients are at high risk for cirrhosis, with up to 40% of patients developing liver fibrosis and up to 20% developing cirrhosis (Altamirano and Bataller, 2011).

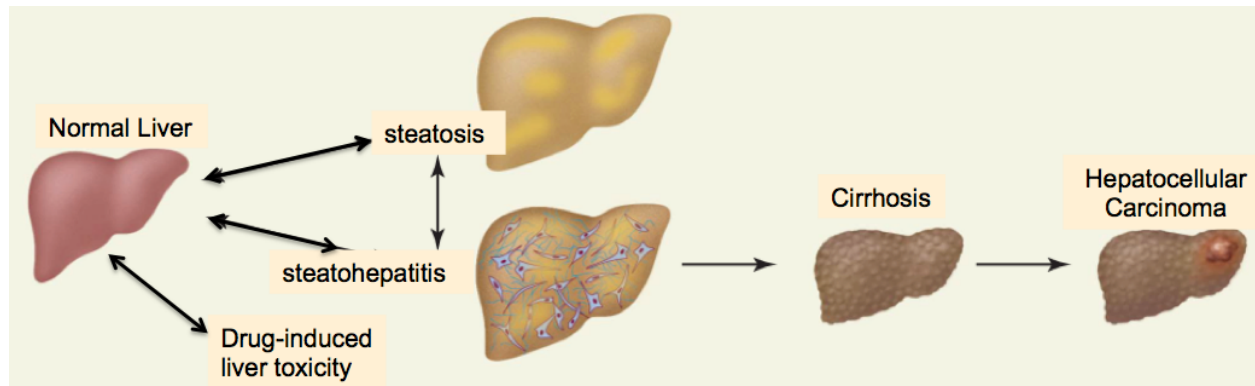


Figure 1-2. Schematic of liver disease progression

Injury to the liver due to nutritional, drug, or alcohol sources can cause steatosis, steatohepatitis or liver inflammation, and acute liver toxicity. Continued exposure to an injury state can result in scar tissue formation (cirrhosis), which impairs proper liver function and predisposes to hepatocellular carcinoma (Adapted from Cohen et al 2007).

It is commonly understood that while a first “hit”, steatosis, disrupts lipid homeostasis in the liver during NAFLD, a second “hit” such as inflammatory cytokine release, oxidative, or endoplasmic reticulum (ER) stress is also required for hepatic steatosis to progress to an inflammatory cirrhotic state (Asaoka et al., 2013; Day and James, 1998; Koteish and Mae Diehl, 2002) (Figure 1-2). An imbalance of gut microbiota resulting from overnutrition may also lead to the presence of bacterial products in the portal circulation, which can act to trigger innate immune responses (Hebbard and George, 2011; Mehal, 2013). Cirrhosis is characterized by fibrosis, proliferative nodules, hepatocyte death, and vascular reorganization, which may lead to liver failure that currently can only be treated by liver transplantation. The combination of increased free fatty acids, inflammatory response, ROS, and autophagy can cooperate to damage hepatocytes, initiate scar tissue formation, even promote malignant transformation (VanSaun et al., 2009).

The most common form of liver cancer, hepatocellular carcinoma (HCC), is the fourth deadliest cancer, with a relative 3-year survival rate of 17% (El-Serag, 2007). The vast majority (up to 80%) of HCC cases arise from cirrhosis, and NAFLD contributes to 58% of HCC patients (Anstee et al., 2013). The estimated stabilization of HCC incidence from viral hepatitis is expected to be superseded by the increase of HCC from NAFLD (El-Serag, 2007). Current research into this disease progression from simple steatosis to cirrhosis and malignant transformation seeks to identify tractable drug targets (Wree et al., 2013).

The genetic factors mediating NAFLD and ALD disease progression are not well understood; however, studies of families and twins, and across ethnicities imply a strong genetic component (Altamirano and Bataller, 2011; Anstee and Day, 2013). Identifying the molecular pathways that cause or modulate disease progression can distinguish the populations at greatest

risk and lead to novel therapies in this area of great clinical need. Animal models of liver disease have proven useful for elucidating gene functions and for drug discovery. Aspects of chronic liver diseases can be modeled in mice and zebrafish using specialized diets such as high fat diet, methionine/choline deficient diet, and administration of ethanol or hepatotoxins such as thioacetamide (Altamirano and Bataller, 2011; Amali et al., 2006; Rekha et al., 2008). Genetic models exhibiting features of hepatic steatosis also exist, including the *foie gras* (Sadler et al., 2005) and *dtg*^{+/-} mutant zebrafish, the leptin deficient *ob/ob* mice, or other mice lacking genes important for carbohydrate and lipid metabolism or the inflammatory response (Anstee and Goldin, 2006; Hebbard and George, 2011; Koteish and Mae Diehl, 2002).

Zebrafish as a model organism

Determination of disease mechanisms requires an experimental system that closely recapitulates the interaction between complex tissues and environments. Furthermore, drug discovery can be made more informative and applicable to humans when using *in vivo* animal models rather than isolated cell populations. The zebrafish (*Danio rerio*) has been an important vertebrate model for elucidating the mechanisms of organogenesis and disease pathogenesis. Developmental and physiological programs in zebrafish and mammals are highly conserved, with 70% similarity (Howe et al., 2013) between zebrafish and humans. Unlike mice, zebrafish breeding strains are outbred populations, which allow for assessment of phenotypic variation across defined populations and relevance to human genetic variation. Examples of human diseases that can be modeled in zebrafish include developmental defects, liver disease, infectious disease, hematopoietic malignancies, and solid tumors (Berghmans et al., 2005; Haramis et al., 2006; Lieschke and Currie, 2007; Patton et al., 2005; Tobin et al., 2010).

Several experimental features make zebrafish an attractive model organism. Hundreds of embryos can arise from a single mating per week, and these embryos are optically clear, leading to efficient analysis of large numbers simultaneously. Embryogenesis proceeds on a faster time scale in zebrafish compared to mammals. Because embryonic development occurs externally, visualization of developmental milestones can occur *in vivo*, and use of transgenic fluorescent reporters have led to many insights when employing live visualization of organs or even individual cells. Furthermore, transgenic reporters and genome editing technology such as zinc finger nucleases (ZFN), transcription activator like effector nucleases (TALEN), and the clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 system can be applied to the analysis of normal and aberrant states in zebrafish.

The first large-scale genetic screens using vertebrates were conducted with zebrafish and yielded a variety of mutants to inform essential developmental processes such as germ layer specification and organogenesis (Driever et al., 1996; Haffter et al., 1996). Since then, zebrafish have proven to be a powerful vertebrate model for both genetic and chemical screening, which have emerged as effective methods to generate mutants, discover novel pathways, and test drug efficacy (Lieschke and Currie, 2007; Patton and Zon, 2001). Beyond screening for developmental defects, newer technologies allow for screening to include fluorescent reporters and modulation of disease phenotypes.

The zebrafish liver performs the same functions as the mammalian liver and is subject to similar injury induction procedures that can be modified experimentally using chemical and genetic tools. An advantage of modeling liver disease in zebrafish is that injury can be studied in both the larval and adult stages. Larval studies allow for high-throughput testing and rapid experiment times. Alcoholic steatosis, acetaminophen-induced liver toxicity, and diet-induced

steatosis are all injury conditions that can be modeled in zebrafish larvae (Farber et al., 2012; North et al., 2010; Passeri et al., 2009; Tainaka et al., 2011). Previous studies have also established partial hepatectomy in zebrafish as a method to discover and assess regulators of adult liver regeneration (Goessling et al., 2008; Sadler et al., 2007).

As zebrafish continue to gain prominence as a model organism for studying developmental processes and for modeling injury and disease, many laboratories have developed experimental tools for the detection and analysis of specific liver cell types. The cell types important for mammalian liver function and injury response are also conserved in zebrafish. The *Tg(fabp10a:GFP)* hepatocyte reporter line has long been used for visualizing liver size and hepatic damage over time (Her et al., 2003). More recently, *hand2*, which becomes upregulated during liver injury, has been identified as the zebrafish hepatic stellate cell marker (Yin et al., 2012). Additionally, fluorescent reporter lines are also available for studying liver endothelial cells (Sakaguchi et al., 2008) and macrophages have been found in the zebrafish liver (Wittamer et al., 2011), but their characterization as bona fide Kupffer cells has not been established. Zebrafish biliary development occurs in parallel with hepatocyte expansion and liver outgrowth. The 2F11 marker and *ck19* transgenic reporter (Crosnier, 2005; Wilkins et al., 2014) are tools to visualize biliary epithelial cells, while exposure to digestible fluorophores such as PED6 and BODIPY-C5 (Carten et al., 2011; Farber et al., 2001) allow for *in vivo* analysis of gall bladder structure and function, lipase activity, and lipid metabolism in the biliary system.

Liver development in mammals and zebrafish

Liver disease pathogenesis involves proliferation and recapitulation of genetic pathways normally limited to liver development. Many pathways necessary for organogenesis are frequently disrupted during disease, and this relationship is conserved in zebrafish. For example,

Wnt signaling, as described below, is essential for hepatic specification and outgrowth. Its requirement in specific cell types and at specific times is integral for proper liver formation (Goessling et al., 2008). This pathway is frequently mutated or aberrantly expressed in human cancers such as liver and colorectal cancers. Therefore, knowledge of the spatial and temporal actions of important developmental regulators is not only critical for describing key milestones in organ formation, but also provide therapeutic targets for adult diseases when these regulators become perturbed. Another example is prostaglandin E2, which was originally implicated in zebrafish as an important regulator of hematopoietic stem cell proliferation. This molecule is now in human clinical trials as a potential drug for improving the outcome of umbilical cord blood transplantation (Goessling et al., 2011; North et al., 2007). Additionally, leflunomide is a drug previously approved for rheumatoid arthritis and was discovered in a zebrafish screen for inhibitors of neural crest development. This drug was found to be effective in zebrafish and murine models of melanoma, which is a cancer of the neural crest lineage, and its effectiveness is now being tested in clinical trials to treat patients with advanced melanoma (White et al., 2011).

The liver derives from the endoderm, which gives rise to the gastrointestinal and respiratory tracts in vertebrates. During gastrulation in mice, the transforming growth factor (TGF)- β signaling molecule Nodal functions by embryonic day 7 (e7) to delineate between endodermal and mesodermal fates in a concentration-dependent manner, with higher concentrations inducing endodermal fate (Zorn, 2008). *Sox17* and *FoxA1-3* act downstream of Nodal and are essential for endoderm induction (Fukuda and Kikuchi, 2005). During early somitogenesis, FGF and Wnt signaling gradients pattern the endoderm along the anterior-posterior axis, with repression of Wnt leading to formation of the foregut, which is the future site of the liver (Zorn, 2008). Hepatic induction is initiated from FGF and BMP signals in the

mesoderm, while transcription factors *Hhex*, *Prox1*, and *Gata4,6* are required for hepatoblast specification (Si-Tayeb et al., 2010). In contrast to early somitogenesis when Wnt represses liver development, Wnt signaling during liver bud outgrowth enhances hepatocyte proliferation and differentiation (Zorn, 2008). Hepatoblasts have bipotential characteristics, with those located near portal veins differentiating into biliary epithelial cells via Wnt, TGF β , and Notch signaling while most other hepatoblasts take on hepatocyte fate (Zorn, 2008). Mouse liver development proceeds throughout embryonic development *in utero* and hepatic maturation continues after birth (Figure 1-3).

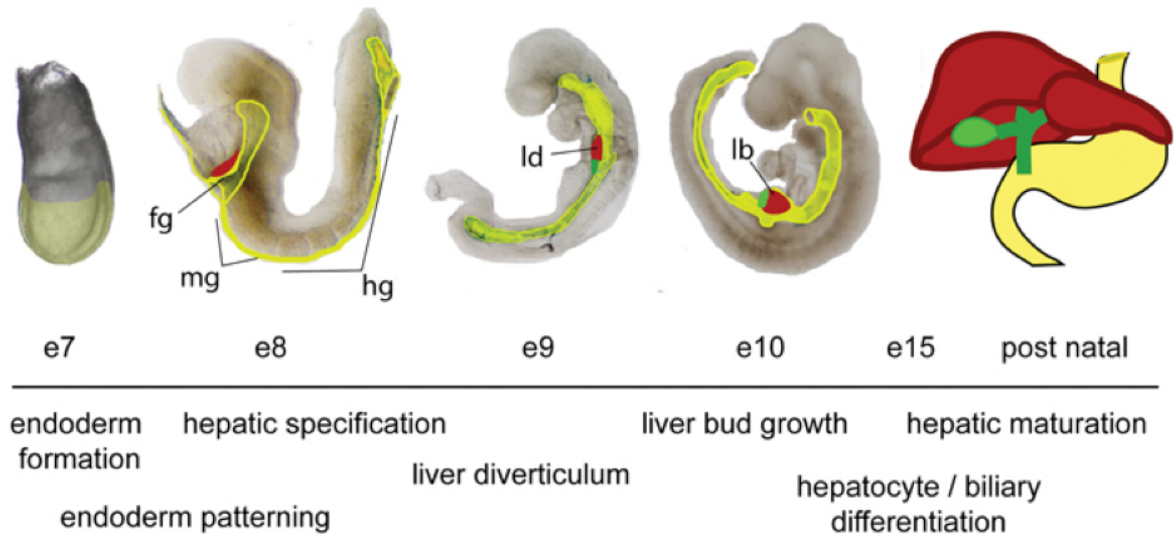


Figure 1-3. Mouse liver development

Mouse liver development begins with endoderm formation at embryonic day 7 (e7), followed by hepatic specification in the foregut endoderm (fg) and development of the liver diverticulum (ld) by e9. Hepatocyte differentiation and liver maturation continues up to birth and postnatally (Zorn, 2008).

This process is conserved in vertebrates, including zebrafish. The endoderm is distinguished in zebrafish through the upstream action of Nodal signals to activate *gata5/fau*, *cas*, and subsequently *sox17*, which is expressed beginning at gastrulation (Alexander and Stainier, 1999; Stainier, 2002). By 24 hours post fertilization (hpf), most organ systems are specified in zebrafish. Specifically, hepatoblasts, or hepatic progenitors, are specified in the anterior foregut endoderm at 22 hpf and can be identified by the expression of *hhex* and *prox1* (Shin et al., 2007) (Figure 1-4). By 48 hpf, hepatocyte and biliary differentiation begin, which can be visualized by the expression of *fabp10a*, a marker of differentiated hepatocytes (Her et al., 2003). The liver bud can be detected on the left side of the dorsal midline, over the yolk. Similar to mice, Fgf, Bmp, and Wnt signals drive hepatogenesis in zebrafish. Wnt induction early in development acts to inhibit liver formation, while later Wnt induction acts to promote hepatic differentiation and proliferation (Chu and Sadler, 2009; Goessling et al., 2008). Furthermore, Notch signaling plays an important role in specifying biliary cells (Lorent, 2004).

The enzymatic and bile excretion functions of the liver are fully functional by 5 days post fertilization (dpf), concurrent with exhaustion of yolk nutrition and transition to feeding on outside energy sources. The growth of the liver is also supported by, but not dependent on expansion of endothelial cell population to form the liver vasculature (Chu and Sadler, 2009; Sakaguchi et al., 2008). Importantly, embryonic hematopoiesis does not occur in the liver, unlike in mammals, so analysis of liver defects can occur without danger of embryonic lethality due to anemia. Although many of the pathways governing early liver formation have been characterized, our understanding of liver associated processes such as embryonic energy utilization is still lacking.

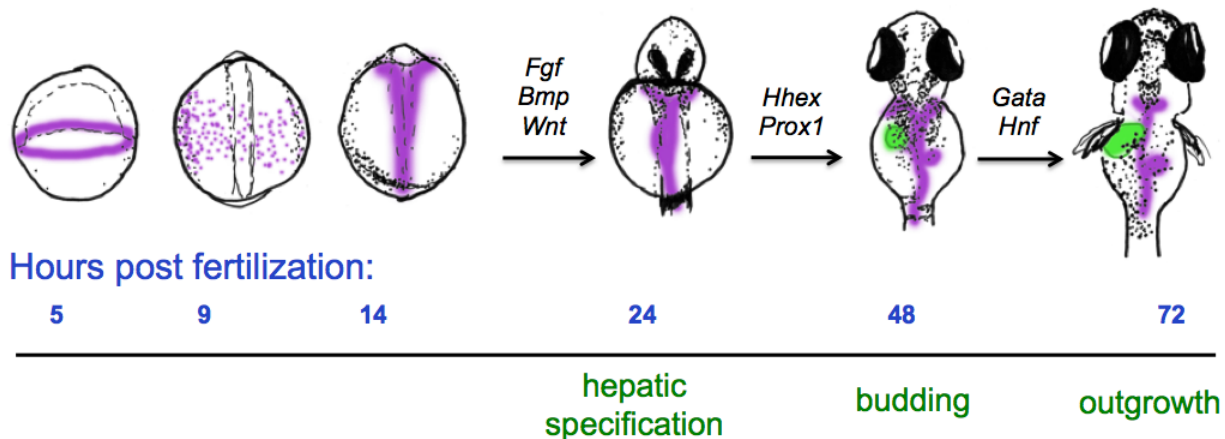


Figure 1-4. Zebrafish liver development

Zebrafish liver development begins with hepatic specification via Fgf, Bmp, and Wnt signals by 24 hours post fertilization (hpf) from the endoderm (purple). Hepatic progenitors can be identified by the expression of *hhex* and *prox1*, and the liver (green) begins to differentiate and bud at 48 hpf, with hepatocyte proliferation and hepatic outgrowth proceeding from 72 hpf onward. Image courtesy of Diane Saunders.

The endocannabinoid signaling pathway

The endocannabinoid (EC) signaling pathway has known roles in liver disease and also influences motor function, appetite, energy balance, cell fate, and immune/inflammatory responses (Caraceni et al., 2009). However, this pathway has no previously established role in liver development. Endocannabinoids are endogenous arachidonic-acid derived lipid signaling molecules that act through the cannabinoid receptors CB1 and CB2, which are encoded by the *cnr1* and *cnr2* genes, respectively (Matsuda et al., 1990; Munro et al., 1993). The endogenous ligands for CB1 and CB2 are 2-arachidonoylglycerol (2-AG) (Sugiura et al., 1995) and anandamide (AEA) (Felder et al., 1993), which are degraded by monoacylglycerol lipase and fatty acid amid hydrolase, respectively (Silvestri and Di Marzo, 2013). The metabolic pathways involving EC synthesis and degradation have been well characterized, although there may be additional EC molecules that have not been discovered. The primary psychoactive ingredient in marijuana, Δ^9 -tetrahydrocannabinol (THC), shows structural similarity to AEA and 2-AG and acts at CB1 and CB2 to produce its cognitive and sedative effects (Guzmán, 2003).

CB1 and CB2 are G-protein coupled receptors (GPCR) that inhibit adenylyl cyclase and decrease cyclic AMP levels while stimulating MAP kinase. Binding to receptors also alters calcium and potassium channel activity, which leads to activation of the ERK, JNK, or PI3K/AKT downstream pathways to control cell fate (Figure 1-5) (Felder et al., 1993; McAllister and Glass, 2002; Pagotto et al., 2006). CB1 and CB2 can mediate distinct downstream effects: CB1 couples to K^+ and Ca^{++} channels to inhibit the release of neurotransmitters whereas CB2 does not (Caulfield and Brown, 1992; McAllister and Glass, 2002). Endocannabinoid signaling also regulates sphingolipid metabolism via sphingomyelin breakdown and increasing levels of ceramide (Figure 1-5). In the central nervous system, EC signaling modulates appetite and

feeding response via retrograde signaling at synapses to inhibit neurotransmitter release (Silvestri and Di Marzo, 2013). The role of cannabinoids in cancer is unclear: in cancer cell lines but not in untransformed cell lines, cannabinoids have been found to induce apoptosis, initiate cell-cycle arrest, or inhibit angiogenesis. Interestingly, in many cases, this occurs in tumors where receptor expression positively correlates with tumor aggressiveness, and inhibition of signaling through CB1 using antagonists can slow tumor growth in several *in vitro* and *in vivo* cancer models (Guzmán, 2003; Pisanti et al., 2013).

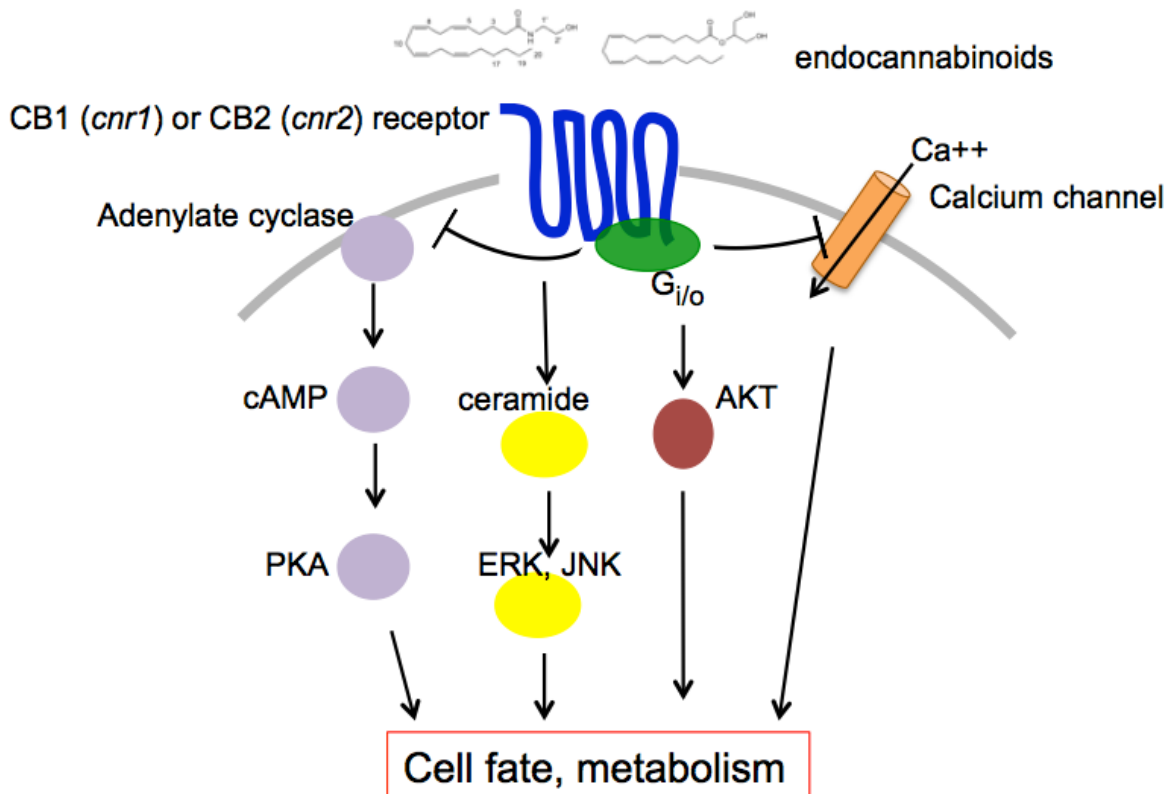


Figure 1-5. Endocannabinoid signaling and activation of downstream pathways

After binding to their receptors, cannabinoids can mediate many downstream pathways to impact cell fate and cellular metabolism, including inhibition of the adenylyl cyclase (AC)-cyclic AMP (cAMP)-protein kinase A (PKA) pathway, inhibition of voltage sensitive calcium channels, activation of the PI3K/AKT signaling, activation of MAP-kinase cascades such as ERK, JNK, p38, and production of ceramide through sphingomyelinase (SMase) to promote apoptosis.

At the tissue level, CB1 is the most abundant GPCR in the central nervous system (CNS) but is also expressed in the peripheral nervous system (PNS) and peripheral organs. CB1 is first expressed in zebrafish at the 3-somite stage (Migliarini and Carnevali, 2009) and exposure to AEA induces expression of CB1 (Migliarini and Carnevali, 2008). In mammals, CB2 is primarily expressed in immune cells (Atwood and Mackie, 2010), and in zebrafish, CB2 is expressed in the adult heart, intestine, spleen, and portions of the CNS (Rodriguez-Martin et al., 2007). There are many chemical modulators of cannabinoid receptor function that have been used in research and as drugs. THC and its derivatives have been approved for treatment of nausea and vomiting in cancer patients undergoing chemotherapy, and have the effect of increasing appetite (Guzmán, 2003). There are many unanswered questions in the EC signaling field, such as how this pathway functions during early development and how signaling through cannabinoid receptors determines cell fate in different cell types.

Endocannabinoids and liver disease

Experiments using chemical modulators and conditional mouse mutants have shown that in addition to CNS effects, the EC system has independent metabolic and injury response functions in peripheral tissues such as the gut and the liver. In mammals, CB1 and CB2 are weakly expressed in the liver under normal conditions (Mallat and Lotersztajn, 2008), but are upregulated during chronic liver diseases such as NAFLD, ALD, viral hepatitis, cirrhosis, and HCC (Caraceni et al., 2009; van der Poorten et al., 2010). CB1 activation induces de novo fatty acid synthesis in the liver, mediated through the central lipogenic transcription factor sterol regulatory element binding protein 1-C, (SREBP-1C) (Osei-Hyiaman et al., 2005b), whereas CB1^{-/-} mice are resistant to diet and ethanol-induced steatosis (Jeong et al., 2008; Osei-Hyiaman et al., 2008). In mammals and zebrafish, CB1 activation in the liver drives SREBP-1C to

increase expression of fatty acid synthase, acetyl CoA carboxylase, and other enzymes that increase fatty deposition (Migliarini and Carnevali, 2008). In addition to its lipogenic roles, CB1 also regulates pro-fibrogenic pathways and is highly upregulated during fibrosis in hepatocytes (Osei-Hyiaman et al., 2005a) and in non-parenchymal liver cells (Teixeira-Clerc et al., 2010). Previously, the CB1 antagonist rimonabant was found to reduce weight and improve liver enzyme levels in obese and diabetic human patients (Kaser et al., 2010). Unfortunately, drug trials in Europe revealed increased depression and suicide risk due to rimonabant action in the CNS, leading to the removal of this drug from the market (Izzo and Sharkey, 2010). However, newly-developed peripherally-restricted CB1 drugs may circumvent this problem (Tam et al., 2010), or alternatively, modulating the CB2 receptor may also decrease susceptibility or severity of metabolic liver disease.

Whereas CB1 activation is pro-fibrogenic and favors liver disease progression, CB2 activation inhibits disease processes by decreasing inflammation, proliferation, and oxidative stress (Bátkai et al., 2007; Julien et al., 2005) (Figure 1-6). CB2 is also upregulated in liver disease; administration of CB2 agonists results in decreased inflammation, decreased fibrosis, and a reduction in activated HSCs in a mouse model of cirrhosis (Muñoz-Luque et al., 2008). Hepatotoxin administration and partial hepatectomy in CB2^{-/-} mice result in greater liver injury and delayed liver regeneration while treatment with CB2 agonists results in reduced injury and enhanced regeneration (Teixeira-Clerc et al., 2010). CB1 and CB2 appear to have opposing roles in the same disease process; however, CB1 effects may predominate in liver pathogenesis, since frequent marijuana users display liver fibrosis and THC acts on both receptors (Hézode et al., 2005; Siegmund and Schwabe, 2008).

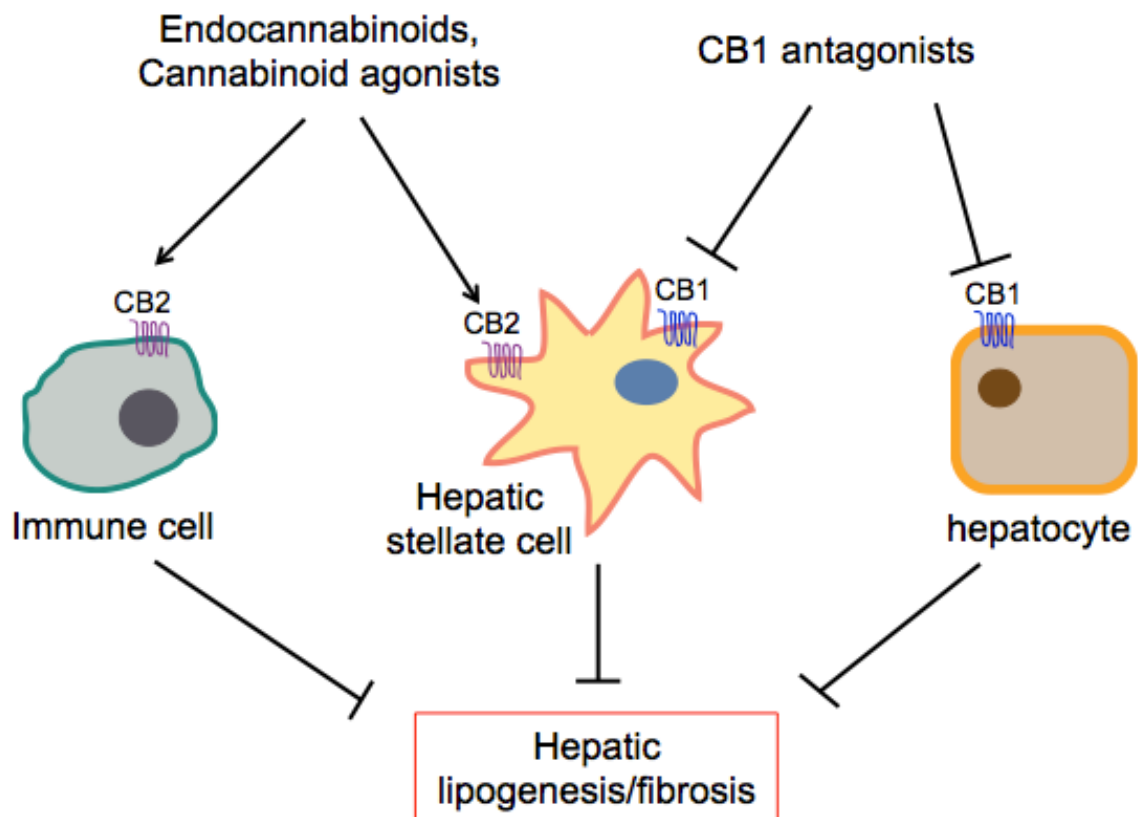


Figure 1-6. CB1 and CB2 functions in liver disease

Studies using mouse models and chemicals have implicated signaling through CB1 in promoting liver lipogenesis and fibrosis. In contrast, signaling through CB2 may ameliorate or prevent fat accumulation and scar tissue formation. Cannabinoid agonists act on CB2 in liver immune cells and hepatic stellate cells while cannabinoid antagonists inhibit signaling through CB1 in hepatocytes and hepatic stellate cells to improve liver disease features.

Summary

In the ensuing chapters, we apply two parallel experimental approaches for examining the intersection of liver development and disease. Chapter 2 explores the role of endocannabinoid signaling in hepatic maturation and analyzes the molecular and metabolic regulators of this pathway as the liver matures. The CB1 and CB2 receptors are expressed in liver tissue and have divergent roles in mediating hepatic lipogenesis and fibrogenesis. However, the role of the EC pathway in embryonic development, specifically liver specification and growth, is unknown. Our lab has successfully modeled aspects of liver injury and used zebrafish to screen for novel compounds and pathways that modulate liver disease (North et al., 2010). We previously conducted a chemical screen that spanned the endoderm specification, hepatoblast differentiation, and hepatocyte proliferation stages of hepatogenesis to identify novel regulators of liver development. We exposed the *Tg(fabp10a:GFP)* liver reporter line to a library of ~2600 bioactive compounds and found that 8.7% of the compounds increased liver size and 5% decreased liver size (Figure 1-7). Many of the molecular pathways identified by this chemical screen such as retinoic acid signaling, nitric oxide signaling, and prostaglandins were found to be important for endoderm and hepatic formation (Cox et al., 2013; Garnaas et al., 2012; Goessling et al., 2009; Nissim et al., 2014; North et al., 2010). Eicosanoid modulators represented 18% of the hits from the screen, and five of these compounds were endogenous or exogenous cannabinoid agonists that all increased liver size in *Tg(fabp10a:GFP)* fish (Figure 1-8). This approach identifies a novel developmental pathway using a zebrafish model and our work in Chapter 2 reveals new insights into its molecular mechanisms with relevance for human liver physiology and disease susceptibility.

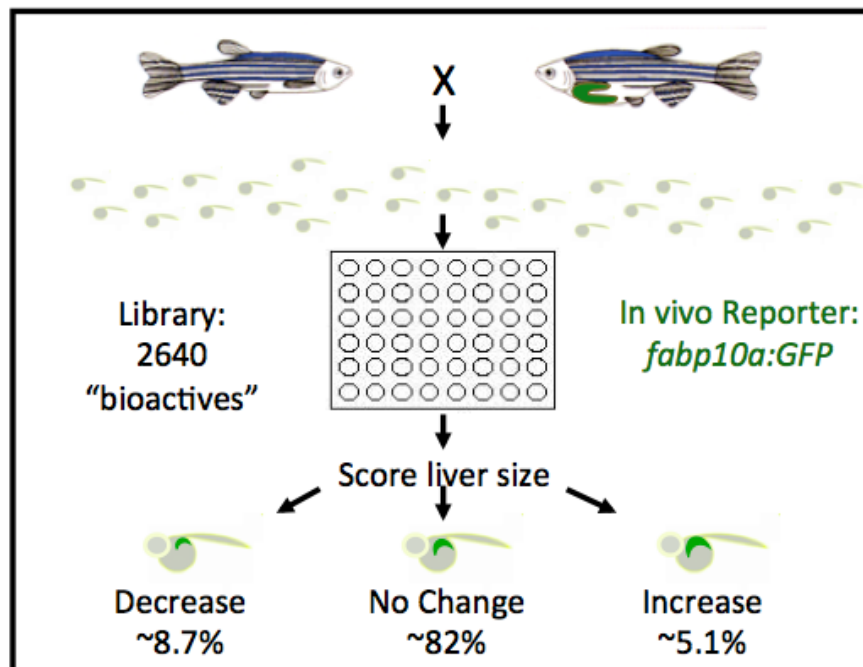


Figure 1-7. Schematic of chemical screen

Tg(fabp10a:GFP) fish expressing GFP under a liver specific promoter were exposed to a library of 2640 chemicals from 18-72 hours post fertilization (hpf). At 72 hpf, 8.7% of the compounds were found to decrease liver size and 5.1% increased liver size.

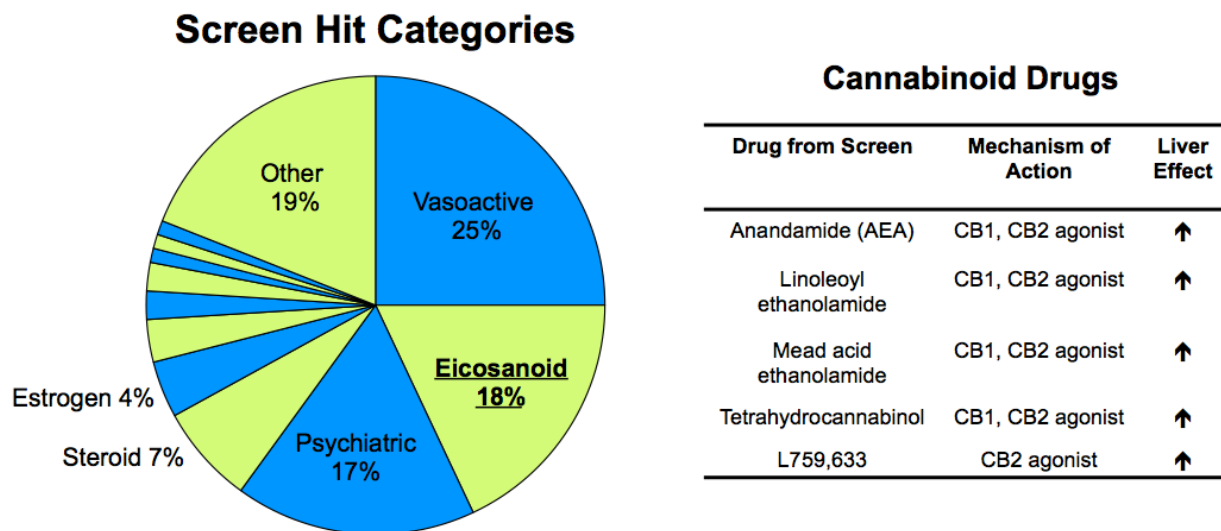


Figure 1-8. Chemical screen hits

Compounds from the screen that modulated liver size were grouped into common pathways, with vasoactive, eicosanoid, and psychiatric chemicals representing the majority of the hits.

Eicosanoid modulators represented 18% of the compound hits, and the five cannabinoid agonists from the screen that all increased liver size are listed in the table at right.

Conversely, in Chapter 3, we undertook a different approach to identify novel regulators of liver development and began with a human genomic data set. We analyzed genome-wide association study (GWAS) data for candidates associated with elevated liver enzyme levels (Chambers et al., 2011). Although candidate gene studies have linked certain genetic variations to liver disease traits, GWAS allow for an unbiased assessment of traits in large populations to discover previously uncharacterized associations. Novel genetic associations often have previously unknown biological functions or mechanisms, necessitating extensive follow-up to establish biological importance. Compared to inbred rodent models, wild type zebrafish are outbred populations and allow for analysis of population-wide phenotypic variation that is found in humans. This system provides initial data for additional analysis of specific candidates, so resource-intensive experiments can be focused to genes most likely to have an important biological function.

These two chapters represent complementary ways a vertebrate model can be used to study the biological and disease relevance of novel regulators. The zebrafish can play an integral role in the translational application of genomic data from population-based studies: discoveries made in humans via GWAS can be validated and interrogated in zebrafish or other model organisms to gain insight into biological mechanisms that can be applied back to human disease.

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Chapter 2: Cannabinoid receptors regulate hepatic differentiation and metabolism during development

Cannabinoid receptors regulate hepatic differentiation and metabolism during development

Leah Y. Liu¹, Bani Mukhopadhyay², Resat Cinar², George Kunos², Trista E. North^{3,4}, Wolfram Goessling^{1,4,5,6,7}

¹ Genetics Division, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, USA

² Laboratory of Physiological Studies, National Institute on Alcohol Abuse and Alcoholism, Bethesda, MD 20982, USA

³ Department of Pathology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02115, USA

⁴ Harvard Stem Cell Institute, Cambridge, MA 02138, USA

⁵ Gastroenterology Division, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, USA

⁶ Dana-Farber Cancer Institute, Boston, MA 02215, USA

⁷ Broad Institute of MIT and Harvard, Cambridge, MA 02142, USA

This chapter contains a manuscript in revision at *Development*. It has been modified to fit the style of this dissertation. Supplemental data, additional, and ongoing experiments can be found in the Appendices.

Author Contributions: L.Y.L and W.G. conceived and designed the experiments and analyzed the data. L.Y.L performed the zebrafish experiments and wrote the manuscript. B.M., R.C., and G.K. performed endocannabinoid measurements and shared mouse RNA sequencing data. All authors reviewed and edited the manuscript.

Abstract

Endocannabinoid (EC) signaling is best known to mediate psychotropic effects and regulate appetite. In contrast, its roles in organ development and embryonic energy consumption remain unknown. Here, we demonstrate that genetic or chemical inhibition of cannabinoid receptor activity disrupts liver development and metabolic function in zebrafish, impacting differentiated hepatocytes but not hepatic specification: loss of EC signaling leads to smaller livers with fewer hepatocytes and impaired lipid handling. In contrast, EC agonists enhance liver growth during development. While liver mass recovers in cannabinoid receptor mutants, metabolic abnormalities persist throughout larval stages and adulthood. Metabolomic analysis reveals reduced content of methionine and its metabolic intermediates in receptor mutants. Extracts from mutant livers demonstrate reduced protein methylation. Treatment with exogenous methionine rescues the liver developmental defect, indicating a causal relationship between methionine deficiency and impaired liver growth. Using a candidate approach, we demonstrate that Sterol Regulatory Element-Binding Proteins (SREBPs) mediate the effect of EC signaling on methionine and rescue the liver defect in receptor mutants. Our work discovers a novel function for EC signaling during liver differentiation, and metabolomic analysis establishes a functional relationship between EC, methionine metabolism and SREBP signaling throughout liver development.

Introduction

The energy requirements of a developing embryo influence and depend on the development of essential metabolic organs. The factors that impact energy consumption and metabolism during embryogenesis are not well understood. Defects in metabolic processing or insufficient nutrient utilization can disrupt differentiation and growth of essential organs, and different stages of development from stem cell maintenance to tissue specification may each require implementation of specific metabolic programming (Shyh-Chang et al., 2013; Tennessen et al., 2014). Disruption of developmental metabolism may impact adult organ function, as metabolites may also directly regulate essential acetylation and methylation processes, impacting epigenetic control of development (Shyh-Chang et al., 2013; Sinclair and Watkins, 2014). Impairment of cellular proliferation pathways can disrupt organogenesis, which can inhibit normal physiological functions and lead to long-term consequences for adult metabolic homeostasis. Here, we show that endocannabinoid (EC) signaling is required for normal embryonic liver development and function. As a central metabolic organ, the liver is essential for synthesis of metabolic enzymes and homeostatic control of glucose and lipids. Disruptions in these processes contribute to the development of diabetes, obesity, and chronic liver disease. Elucidating the genetic pathways and mechanisms regulating developmental metabolism and subsequent pathogenic processes can lead to early interventions in individuals with genetic disease predisposition.

The endocannabinoid (EC) signaling pathway has predominantly been explored for its effects in the central nervous system (CNS), such as regulating appetite, mood, and pain (Castillo et al., 2012). In contrast, its function during embryonic development is unknown. The effects of ECs are mainly mediated by two principal G protein-coupled receptors, CB1 and CB2. CB1 is

primarily expressed in the CNS where it promotes appetite control (Maccarrone et al., 2010; Matsuda et al., 1990), while CB2 acts in immune cells and the gastrointestinal tract (Munro et al., 1993). Although cannabinoids have been shown to regulate appetite in the CNS and independently in peripheral tissues, how these receptors influence embryonic metabolism and liver function via downstream targets has not been studied.

In this study, we discover that cannabinoid receptors CB1 and CB2 are required for normal hepatocyte differentiation during embryonic development in the zebrafish. Disrupted EC activity leads to diminished and immature hepatocytes, impaired lipase activity and biliary lipid excretion. EC signaling orchestrates critical metabolic functions during early nutritional transitions, and the aberrations in liver physiology in receptor mutants persist in larval and adult zebrafish. Polar metabolomics analysis reveals dysregulation of methionine and its metabolic intermediates as a consequence of disrupted EC signaling. This process is mediated by *SREBP* genes, and both supplementation with methionine and overexpression of *SREBPs* rescues the developmental liver defects in zebrafish larvae. Our work uncovers a previously unrecognized relationship between EC signaling, liver development, and the regulation of metabolic homeostasis during development.

Results

Inhibition of signaling through CB1 and CB2 receptors impacts liver development

We conducted a chemical screen in zebrafish to identify novel regulators of liver development (Garnaas et al., 2012). Cannabinoid agonists increased liver size, as determined by transgenic hepatocyte reporter *fatty acid binding protein 10a (fabp10a):GFP* expression at 72 hours post fertilization (hpf) (Figure 2-1, A). To confirm the screen results, we exposed zebrafish embryos to CB1 and CB2 agonists and antagonists from 18-72 hpf at a concentration of 1 μ M, as

determined by a dosage curve, followed by analysis of liver size at 72 hpf using *in situ* hybridization (ISH) for *fabp10a*. Compared to DMSO-treated control, the nonselective CB1/CB2 agonists O2545 (Martin et al., 2006), the CB1 agonist Leelamine hydrochloride (L-HCl), and the CB2 agonist JWH015 increased liver size, while the antagonists Rimonabant and AM630, targeting CB1 and CB2 respectively, diminished liver formation (Figure 2-1, B-C). These chemical studies suggest an important role for EC signaling in liver development.

Expression of zebrafish receptor homologs *cnr1* and *cnr2* is conserved in the liver and CNS during development at 24, 48, and 72 hpf, indicating the ability of the liver primordium to respond to EC agonists (Figure S2-1, in appendix). To genetically confirm the role of cannabinoid receptors during liver development, we generated *cnr1*^{-/-} and *cnr2*^{-/-} knockout zebrafish by constructing transcription activator-like effector nucleases (TALENs) (Sander et al., 2011) targeting the first exon of the *cnr1* and *cnr2* genes (Figure S2-2, A-B, in appendix).

To avoid possible off-target effects of TALENs, animals were outcrossed for at least 3 generations. *cnr1*^{-/-} and *cnr2*^{-/-} animals survived to adulthood, were fertile, and exhibited no gross morphological defects during development. Compared to wild-type embryos, liver size in both homozygous receptor mutants was significantly decreased at 72 hpf, as assessed by *fabp10a in situ* hybridization, and consistent with the chemical studies (Figure 2-1, D). Heterozygous mutants did not exhibit any liver size differences compared to wild type sibling controls (Figure S2-2, C, in appendix). Changes in liver size and hepatic gene expression were confirmed and quantified by measuring liver morphometrics in micrographs after ISH (Figure 2-1, E) and quantitative PCR (qPCR) for *fabp10a*, revealing a 50% decrease (Figure 2-1, F). Quantification of GFP-positive hepatocytes by FACS analysis in drug-treated and morpholino-injected *Tg(fabp10a:GFP)* reporter embryos demonstrated an increased cell number with agonist

treatment, while hepatocytes were diminished after chemical or genetic inactivation of *cnr1* or *cnr2* (Figure S2-2, D-E, in appendix). These findings were further corroborated by quantification of *cnr1* and *cnr2* morphant liver sizes in the *Tg(fabp10a:GFP)* hepatocyte reporter line, revealing consistent phenotypes and smaller livers at 72 hpf (Figure S2-2, F, in appendix). These combined results from chemical modulation, morpholino knockdown, and engineered mutants establish that *cnr1* and *cnr2* are required for normal liver development in zebrafish.

Figure 2-1 (next page). Chemical and genetic modulation of endocannabinoid signaling alters liver size during development

A) Cannabinoid receptor agonists from chemical screen that increased liver size.

B) Liver size distribution across clutches of zebrafish embryos treated with 1 μ M of CB agonists and antagonists from 18-72 hpf, as assessed by visual inspection. Treatment with agonists shifted the liver size distribution toward “large” livers while treatment with antagonists shifted the liver size distribution toward “small” livers.

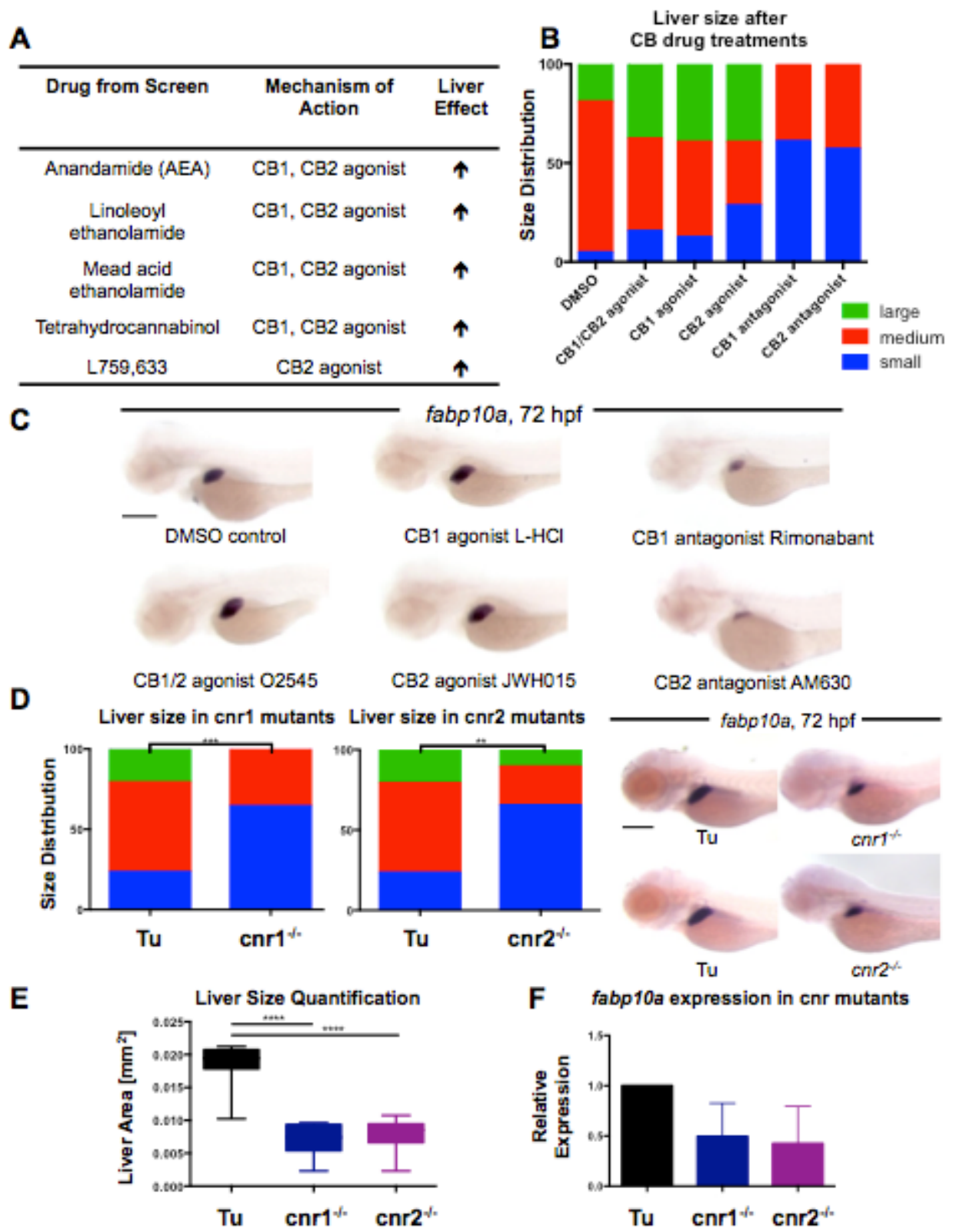
C) *in situ* hybridization for *fabp10a* in zebrafish embryos at 72 hpf after treatment with 1 μ M of CB drugs from 18-72 hpf. Scale bar = 0.2 mm.

D) Liver size distribution and representative *in situ* hybridization images showing decreased *fabp10a* expression in *cnr1*^{-/-} and *cnr2*^{-/-} mutants. Chi-squared analysis, n>50 embryos, ***p<0.001 compared to *cnr1*^{-/-} and **p<0.01 compared to *cnr2*^{-/-}. Scale bar = 0.2 mm.

E) Quantification of liver size in *cnr1*^{-/-} and *cnr2*^{-/-} mutants based on liver morphometric measurements using ImageJ. Data are represented as mean \pm s.e.m with one-way ANOVA analysis, n > 30 samples, ****p<0.0001 for wild-type vs. *cnr1*^{-/-} and wild-type vs. *cnr2*^{-/-}.

F) qPCR of the *fabp10a* gene showing decreased *fabp10a* expression in cannabinoid receptor mutants.

Figure 2-1 (continued)



To determine if the effects of *cnr1* and *cnr2* loss were specific to the liver, we examined other endodermal organs and proximal mesodermal organs: Intestinal development was also disrupted in *cnr1* and *cnr2* mutants, based on *fabp2* expression at 72 hpf; however, exocrine pancreas development, as assessed by *trypsin* expression, was not affected, nor were markers for kidney (*pax2a*) and heart (*cmlc2*) (data not shown). To further delineate the temporal importance of EC signaling during the different stages of endoderm formation and differentiation, embryos were exposed to cannabinoid agonists from 18-24 hpf (endoderm specification), 24-48 hpf (hepatoblast population), or 48-72 hpf (hepatocyte differentiation and proliferation) (Figure 2-2, A). Only exposure to cannabinoid agonists from 48-72 hpf impacted liver size (Figure 2-2, B-C), and expression of the pan-endodermal marker *foxA3* and the hepatoblast marker *hhex* at 48 hpf was unchanged in *cnr1*^{-/-} and *cnr2*^{-/-} mutants or morphants (Figure 2-2, D-F), indicating that the formation and growth of differentiated hepatocytes are impacted by lack of cannabinoid receptors, but earlier stages of endoderm development are not affected. Together, these results reveal that EC signaling is required for hepatocyte differentiation, but not earlier stages of liver development.

Figure 2-2 (next page). Hepatocyte development is disrupted in cannabinoid receptor mutants without impairment of hepatic progenitors

A) Schematic of drug exposure timeline to target the endoderm, hepatoblasts, and hepatocyte cell populations in the developing zebrafish embryo.

B) Relative frequency of liver size distribution of zebrafish embryos treated with CB agonists during different stages of liver development. Treatment with both the CB1/CB2 agonist O2545 1 μ M and with the CB2 agonist JWH015 1 μ M from 48-72hpf increased liver size, while earlier treatment had no effect. Embryos were scored based on *fabp10a* expression. Chi-squared analysis, n>30 embryos, O2545: ***p<0.001 compared to 48-72 hpf treatment group. JWH015: *p<0.05 compared to 48-72 hpf treatment group.

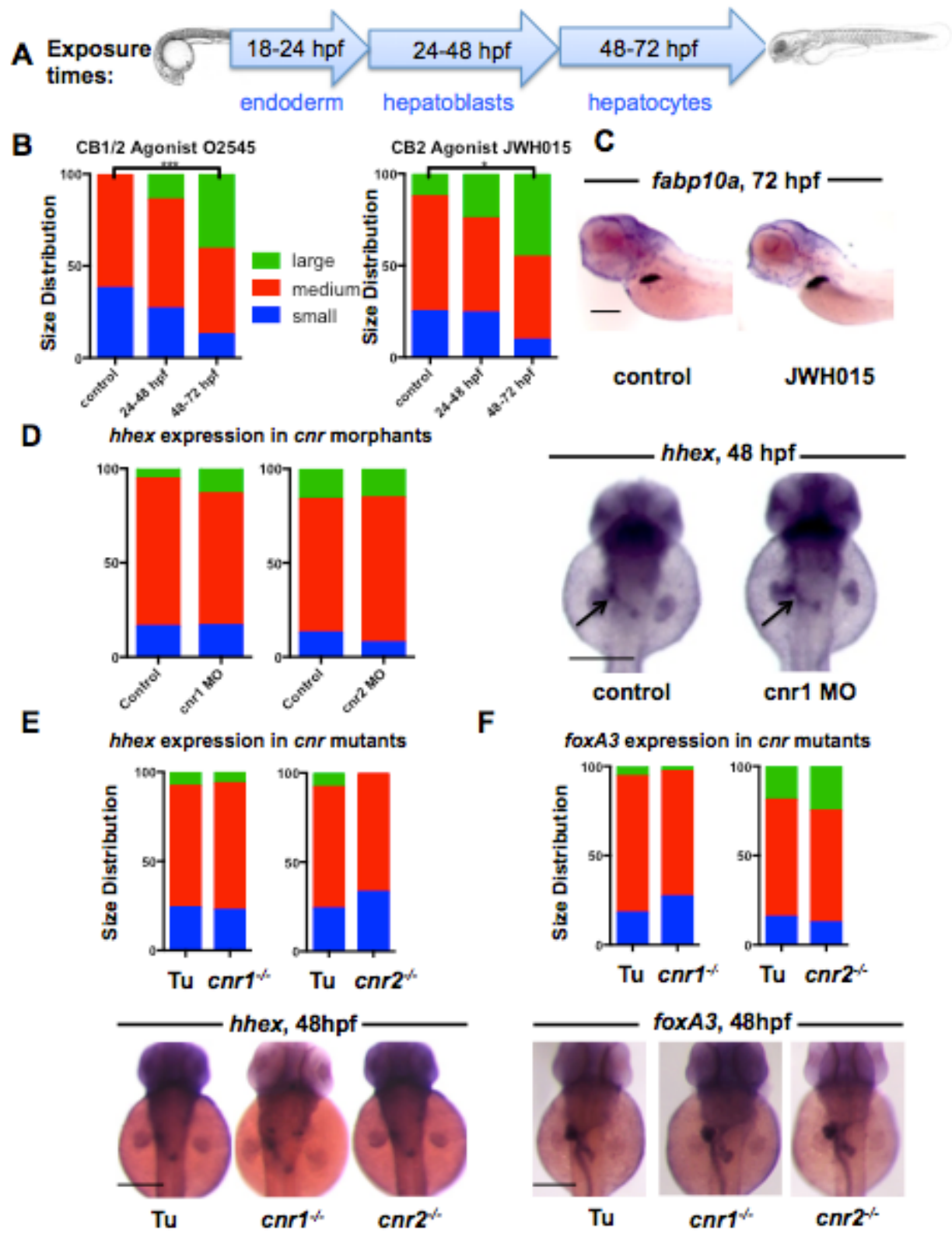
C) Treatment with JWH015 from 48-72 hpf increases *fabp10a* expression in zebrafish embryos at 72 hpf. Scale bar = 0.2 mm.

D) Graph and representative *in situ* hybridization images showing the size distribution of *hhex* expression after morpholino knockdown of *cnr1* and *cnr2*. Morphant embryos do not show differences in *hhex* liver progenitor expression. Chi-squared analysis, n>30, p=NS. Scale bar = 0.2 mm.

E) Graph and representative *in situ* hybridization image of the size distribution of *hhex* expression in *cnr1*^{-/-} and *cnr2*^{-/-} mutants showing no difference in the hepatic progenitor population. Chi-squared analysis, n>30, p=NS. Scale bar = 0.2 mm.

F) Graph and representative *in situ* hybridization image of the size distribution of *foxA3* expression in *cnr1*^{-/-} and *cnr2*^{-/-} mutants showing no difference in the endoderm population. Size distribution comparisons were made using Chi-squared analysis, n>30, p=NS. Scale bar = 0.2 mm.

Figure 2-2 (continued)



cnr1^{-/-} and cnr2^{-/-} mutants exhibit disrupted liver function

In order to examine whether the impact on liver differentiation persisted beyond the initial stages of liver growth, liver size was examined in larval stages: liver size defects in *cnr* mutants are still observed at 96 hpf, but are diminished by 120 hpf (Figure S2-3, A-B, in appendix). To determine if the effect of *cnr1* and *cnr2* loss is restricted to liver size or cell number, we assessed liver cellular morphology in *cnr1^{-/-}* and *cnr2^{-/-}* mutants in histological sections at 120 hpf. *cnr1^{-/-}* and *cnr2^{-/-}* mutant hepatocytes show a more rounded and less mature appearance; also, the intestinal epithelium appears disorganized (Figure 2-3, A). PCNA expression was decreased in *cnr1^{-/-}* and *cnr2^{-/-}* mutants compared to wild type, indicating that reduced cell proliferation is responsible for the liver size differences (Figure S2-3, C, in appendix). Together, these data indicate that even though *fabp10a* expression in mutants at 120 hpf has recovered, histological features remain abnormal into larval stages with cannabinoid receptor loss, consistent with the observed impact on liver differentiation.

In order to determine whether EC signaling also affects other hepatic cell types, we investigated expression of the biliary epithelial marker 2F11 in 120 hpf larval sections. Wild-type larvae exhibit compact and evenly distributed biliary epithelial cells; in contrast, *cnr1^{-/-}* and *cnr2^{-/-}* mutants showed impaired biliary tree formation with more clustered, less uniform appearance (Figure 2-3, B). To confirm these abnormalities in biliary morphology *in vivo* and to also visualize hepatic lipid metabolism, we administered BODIPY C5 fluorophore, which labels all organs where metabolites accumulate (Carten et al., 2011): At 144 hpf, wild type larval livers exhibit highly branched bile ducts; in contrast, both *cnr1^{-/-}* and *cnr2^{-/-}* mutant larvae are characterized by decreased branching. In addition, *cnr2^{-/-}* mutants have accumulated lipid droplets in the liver, indicative of abnormal lipid handling (Figure 2-3, C). To further

characterize the role of EC signaling in lipid metabolism in the developing liver, we used the lipase reporter PED6: lipase-mediated cleavage of PED6 unquenches the fluorophore and results in a fluorescent signal in the intestine and gallbladder (Farber et al., 2001). Both *cnr1*^{-/-} and *cnr2*^{-/-} mutants demonstrate reduced lipase activity at 120 hpf, as evidenced by little or no detectable gallbladder fluorescence (Figure 2-3, D). This indicates that the normal metabolic activities of the liver and intestines are disrupted in mutants. Disrupted lipid metabolism, potentially resulting from aberrant development of metabolic organs, indicates that CB1 and CB2 receptors are necessary not only for proper liver size, but also have important physiological functions for metabolic homeostasis. These results further demonstrate the value of zebrafish mutants to assess developmental aspects of liver metabolism and function during larval stages that are not feasible with morpholino studies.

Figure 2-3 (next page). Livers in cannabinoid receptor mutants eventually develop to normal size but continue to exhibit metabolic and functional defects

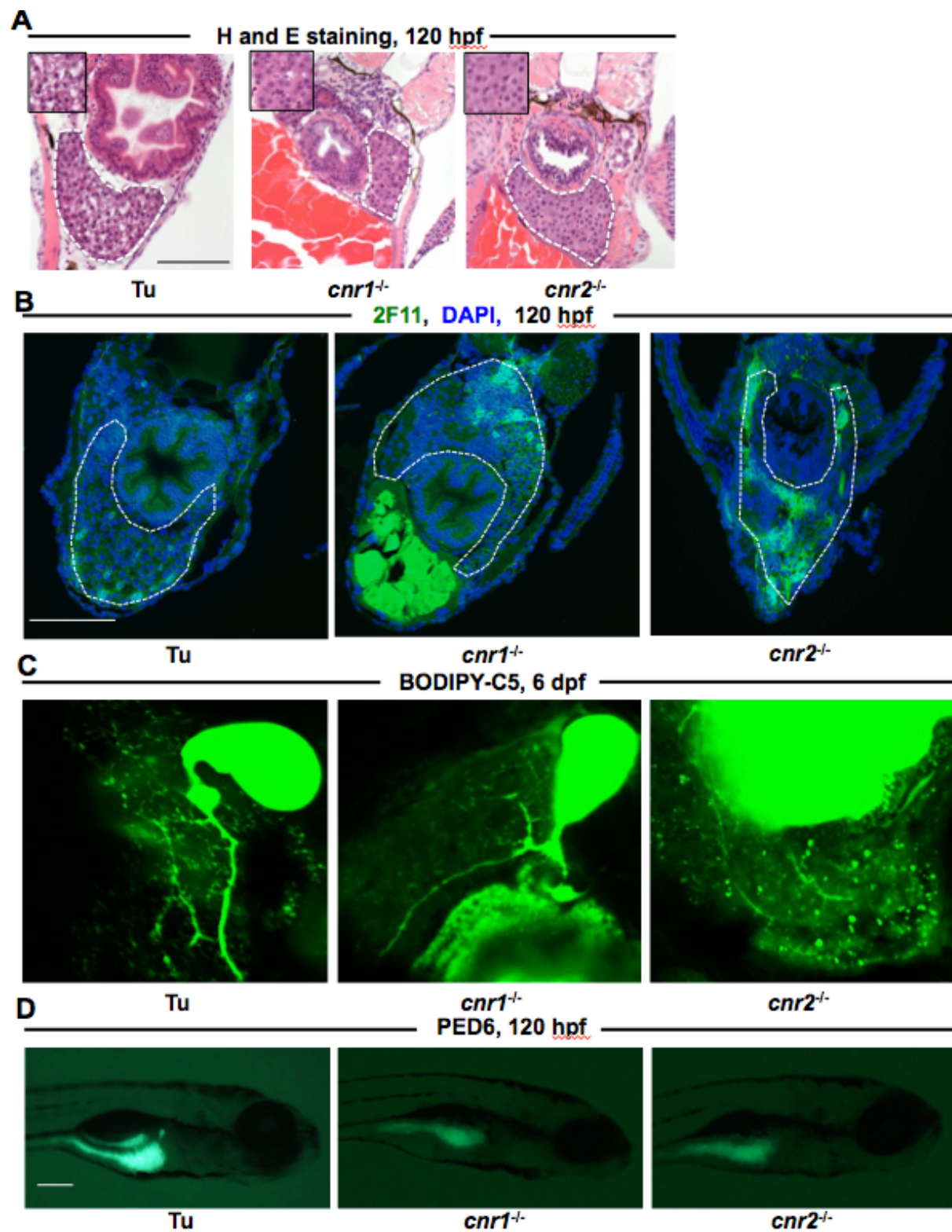
A) H&E-stained transverse sections through 120 hpf larvae show that *cnr1*^{-/-} and *cnr2*^{-/-} mutants have altered hepatocyte morphology. Liver is outlined in white dashes and onset shows magnification of hepatocytes. Scale bar = 0.1 mm.

B) Transverse sections of 120 hpf larvae stained with the biliary marker 2F11 and DAPI show an abnormal biliary tree in *cnr1*^{-/-} and *cnr2*^{-/-} mutants. Scale bar = 0.1 mm.

C) Confocal microscopy images of livers in wild-type and *cnr1*^{-/-} and *cnr2*^{-/-} mutant larvae showing metabolism of the BODIPY C5 fluorophore throughout the liver's biliary tree. *cnr1*^{-/-} larvae have decreased biliary branching while the *cnr2*^{-/-} mutants are characterized by lipid deposits throughout the liver.

D) Examination of PED6 lipase reporter in *cnr1*^{-/-} and *cnr2*^{-/-} mutants shows decreased fluorescent activity. Larvae were scored as “normal” or “abnormal”, and representative images are shown. Scale bar = 0.2 mm.

Figure 2-3 (continued)



cnr1^{-/-} and cnr2^{-/-} mutants demonstrate differential susceptibility to metabolic injury

In order to examine whether the observed changes in lipid handling during larval development also alter susceptibility to pathological levels of lipid accumulation, we used an established protocol (Passeri et al., 2009) to induce alcoholic steatosis. Zebrafish exposed to 2% ethanol from 96-120 hpf develop steatosis at an incidence of 50-60%, which can be visualized using whole mount Oil Red O staining (Figure S2-4, A-B, in appendix). *cnr1^{-/-}*, but not *cnr2^{-/-}* mutants are protected from steatosis, with hepatic Oil Red O staining only detectable in 19% of *cnr1^{-/-}* larvae exposed to ethanol. *hand2*, a marker of zebrafish hepatic stellate cells, which are thought to mediate hepatic injury (Yin et al., 2012), is normally upregulated under liver injury conditions. Consistent with the Oil Red O findings, induction of *hand2* was observed by *in situ* hybridization in *cnr2^{-/-}*, but not *cnr1^{-/-}* 120 hpf larvae exposed to ethanol (Figure S2-4, C-D, in appendix). These findings were confirmed by corresponding observations from morpholino studies and by treatment with CB modulators (Figure S2-4, E-F, in appendix), and are consistent with reported studies on the differential role of CB receptors in steatosis development in mice (Jeong et al., 2008; Louvet et al., 2011a). To determine the impact of EC signaling on nutritionally induced hepatic steatosis, a much bigger clinical problem, we established an egg yolk feeding protocol to mimic a high-fat diet by exposing embryos to 3% egg yolk solution from 96-120 hpf. This treatment induces steatosis in 65% of exposed WT larvae, but in only 24% of *cnr1^{-/-}* larvae (Figure S2-5, A-B, in appendix). Similar to ethanol feeding, co-treatment with CB1 antagonist or CB2 agonist also decreased hepatic steatosis incidence (Figure S2-5, C, in appendix). Our findings indicate that zebrafish mutants at larval stages have aberrant responses to metabolic insult, consistent with reported mammalian biology, further indicating that

developmental disruption of EC signaling affects lipid homeostasis and susceptibility to metabolic insult.

cnr1^{-/-} and cnr2^{-/-} mutants have altered metabolism in adulthood

In order to determine how much the observed developmental and metabolic defects impacted or persisted into adult organ homeostasis and metabolism, we examined adult livers at 6 months. Levels of 2-arachidonoylglycerol (2-AG) (Sugiura et al., 1995) and anandamide (AEA) (Devane et al., 1992), the primary endogenous ligands for CB1 and CB2, in adult liver tissue from *cnr1^{-/-}* and *cnr2^{-/-}* mutant livers were not significantly different from wild-type controls, as determined by liquid chromatography/tandem mass spectrometry (Figure S2-6, A-B, in appendix). This is consistent with findings in the livers of CB1 knockout mice (Mukhopadhyay et al., 2011). Liver mass-to-body-mass ratios in 6-month old wild-type and *cnr1^{-/-}* and *cnr2^{-/-}* mutants were comparable (Figure S2-6, C-D, in appendix). However, liver histology was abnormal, with evidence of cholestasis and inflammatory infiltrates seen in *cnr1^{-/-}* animals, and abnormal cellular morphology, suggesting fatty infiltration in *cnr2^{-/-}* mutants on a regular diet, even in the absence of metabolic injury induction (Figure 2-4, A). Further, expression of the biliary epithelial marker 2F11 was severely diminished in both *cnr1^{-/-}* and *cnr2^{-/-}* mutants (Figure 2-4, B). In order to reveal systemic consequences of altered lipid metabolism, blood was collected from adult zebrafish, which demonstrated significantly elevated triglycerides levels in *cnr2^{-/-}* mutants compared to age-matched *cnr1^{-/-}* mutants and wild-type controls (Figure 2-4, C-D). These observations of abnormal architecture and metabolism in adult animals indicate that disruptions in liver formation or metabolic function at the earliest stages can negatively impact adult homeostasis and global lipid metabolism, even though normal liver size is ultimately achieved.

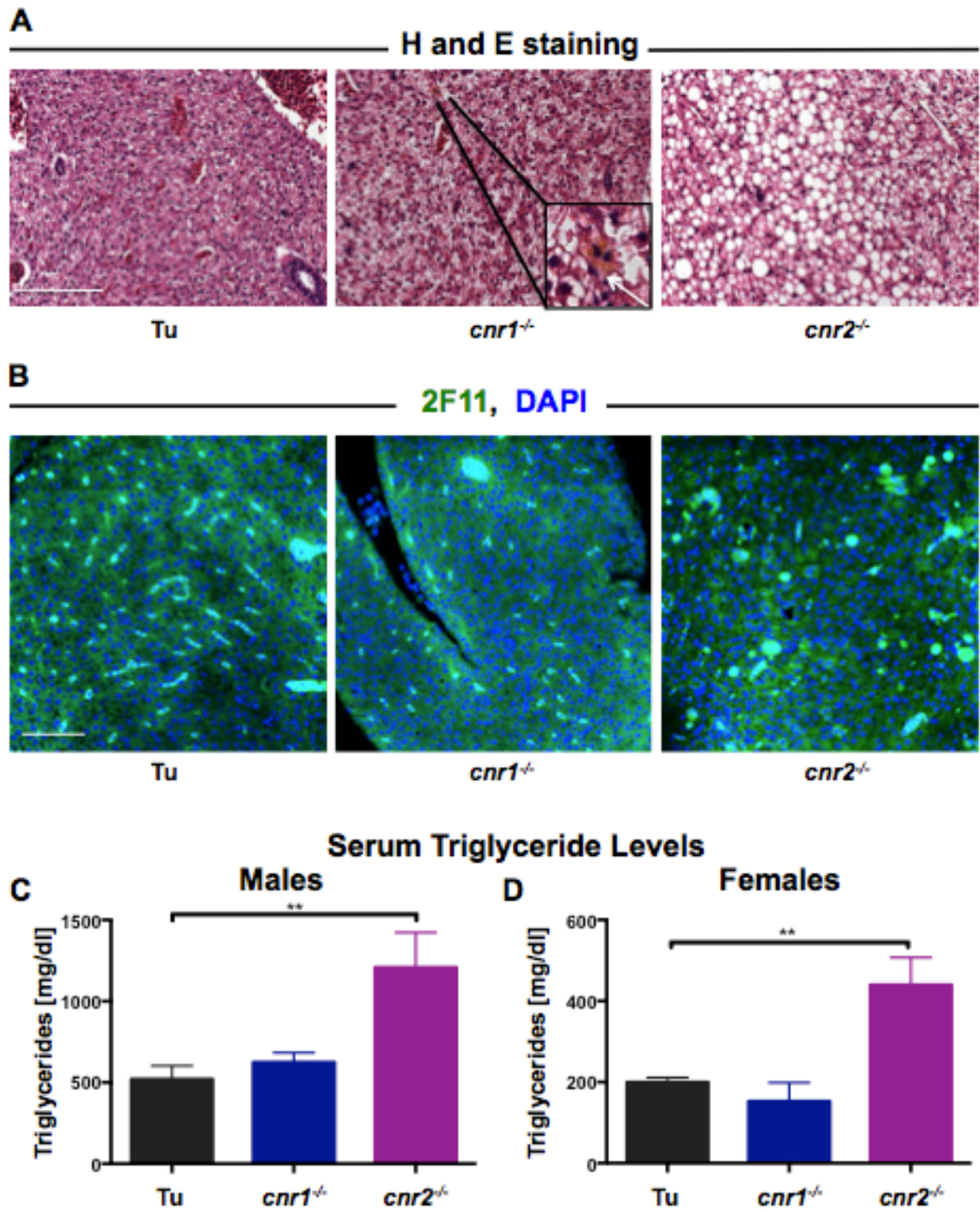
Figure 2-4 (next page). Metabolic dysregulation and aberrant histological features persist in adult cannabinoid receptor mutants

A) H&E-stained adult liver sections show evidence of cholestasis (inset) and inflammatory infiltrates in *cnr1*^{-/-} mutants, and abnormal cellular morphology, suggesting extensive steatosis in *cnr2*^{-/-} mutants. Scale bar = 0.1 mm.

B) Histological sections through adult zebrafish livers stained for the biliary marker 2F11 and DAPI reveal decreased and impaired biliary tree formation in *cnr1*^{-/-} adult zebrafish and cholestatic deposition (arrows) in *cnr2*^{-/-} mutants. Scale bar = 0.1 mm.

C) Quantification of serum triglyceride content in blood collected from male (C) and female (D) cannabinoid receptor mutants. Adult *cnr2*^{-/-} zebrafish have double the triglyceride concentration compared to wild type and *cnr1*^{-/-} fish. Data are represented as mean ± s.e.m, one-way ANOVA analysis, n > 5, **p<0.01 for wild-type compared to *cnr2*^{-/-}.

Figure 2-4 (continued)



Defects in EC signaling affects methionine metabolism

In order to better define the metabolites most affected by disrupted EC signaling and to uncover the metabolic pathways disrupted in *cnr1*^{-/-} and *cnr2*^{-/-} mutants that may be responsible for the observed phenotypes, we pursued metabolomic analysis using mass spectrometry (Yuan et al., 2012) on adult livers, detecting over 290 polar metabolites. Metabolites clustered according to genotype without substantial differences between males and females, revealing several significant changes in individual metabolites from *cnr1*^{-/-} and *cnr2*^{-/-} adult livers (Figure S2-7, A, in appendix). Methionine pathway intermediates were significantly altered between both mutants and wild-type conditions, including reduced levels of methionine, homocysteine, S-adenosyl-homocysteine, cysteine, homoserine, serine, and S-adenosyl-methionine (Figure 2-5, A; Figure S2-7, B-C, in appendix), suggesting defects in methionine metabolism. In order to further determine whether methionine metabolism was substantially affected by disrupted EC signaling, expression of key metabolic enzymes was examined by qPCR. S-adenosylhomocysteine hydrolase (*ahcy*) and spermidine synthase (*srm*) were downregulated in mutant larvae, while methylenetetrahydrofolate reductase (*methfr*) was upregulated (Figure 2-5, B). To confirm genetic conservation of these findings in a mammalian model, we re-analyzed previously published RNA sequencing data from *CB1* knockout mouse livers (Mukhopadhyay et al., 2011). The expression levels of most methionine metabolism pathway enzymes were correspondingly downregulated in *CB1* knockout mice (Figure 2-5, C). These results reveal that loss of either cannabinoid receptor in mice and zebrafish leads to dysregulation of the methionine pathway. Because methionine generates methyl groups for a variety of physiological and molecular functions, we examined global protein methylation levels in 120 hpf larvae or adult livers in *cnr1*^{-/-} and *cnr2*^{-/-} mutants. Levels of methylated protein were diminished in mutant

protein lysate based on detection of methyl-lysine in 120 hpf *cnr1*^{-/-} and *cnr2*^{-/-} mutants, consistent with a global decrease in methionine concentration (Figure 2-5, D). These data indicate that polar metabolomics identified methionine metabolism to be severely impacted in *cnr1*^{-/-} and *cnr2*^{-/-} mutants, which affects global protein methylation status, thereby having potential impact on a multitude of other cellular functions.

Figure 2-5 (next page). Polar metabolomics analysis reveals decreased levels of methionine metabolism intermediates

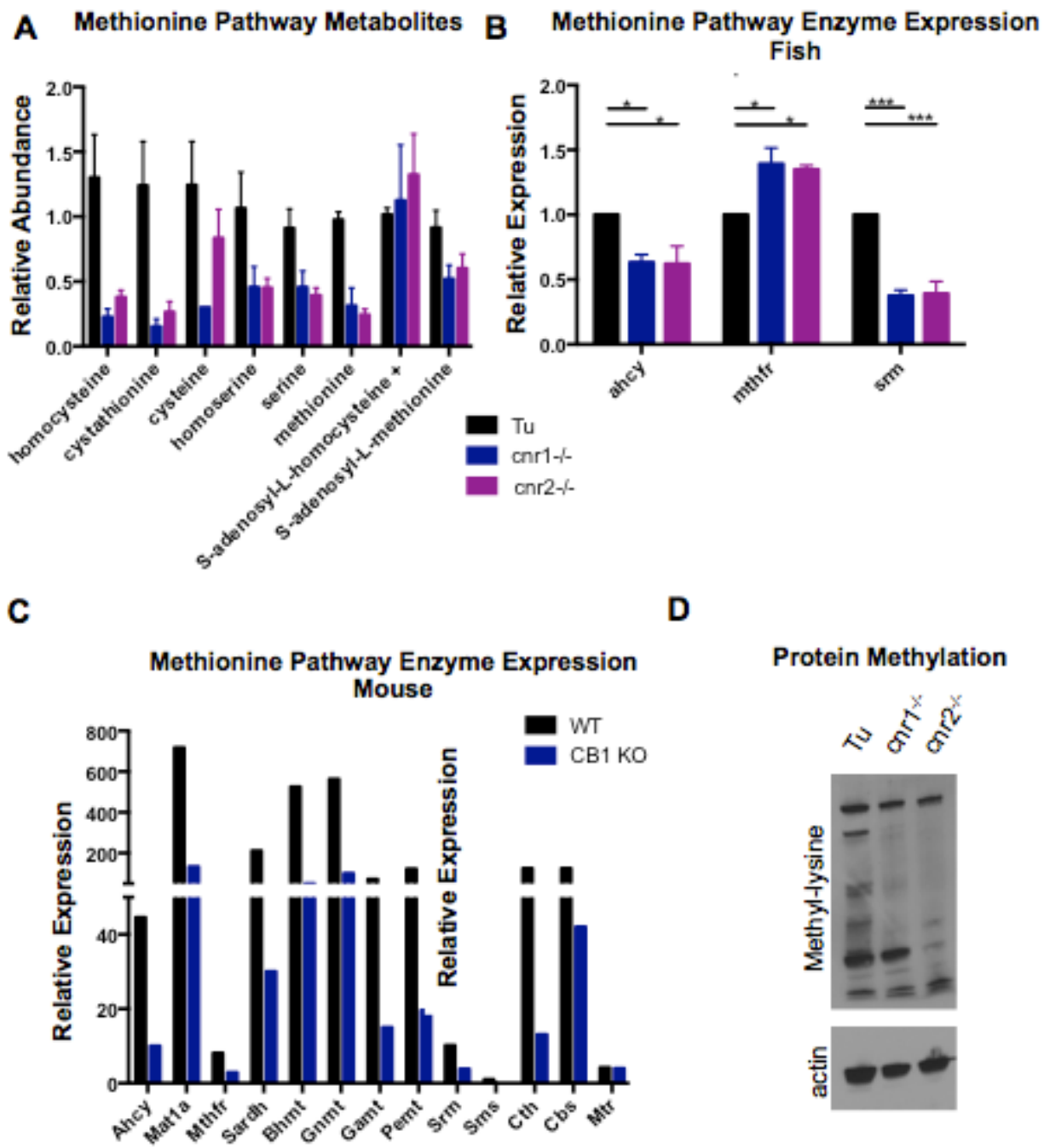
A) Intermediates of the methionine metabolism pathway are decreased in both *cnr1*^{-/-} and *cnr2*^{-/-} mutant adult female liver tissue. Metabolites in male zebrafish showed similar decreases (Figure S2-7, in appedix). Data are represented as mean \pm s.e.m, n=3.

B) Enzymes involved in methionine metabolism are dysregulated in *cnr1*^{-/-} and *cnr2*^{-/-} larvae. *adenosylhomocysteinase (ahcy)* and *spermidine synthase (srm)* are downregulated, while *methfr* is upregulated. n=3, ***p<0.001 and *p<0.05 for expression levels of enzyme in mutant compared to its control.

C) RNA sequencing analysis shows that expression of methionine metabolism enzymes in wild-type compared to CB1 knockout mice is highly dysregulated.

D) Western blot of methylated lysine in total protein from *cnr1*^{-/-} and *cnr2*^{-/-} larvae at 120 hpf shows decreased and altered pattern of methylation in both mutants.

Figure 2-5 (continued)



Methionine rescues liver development defects in cannabinoid mutants

In order to determine if the observed changes in methionine metabolism are directly responsible for the observed liver phenotypes in *cnr* mutants during development, *cnr1*^{-/-} and *cnr2*^{-/-} mutant embryos were exposed to methionine metabolism intermediates. Treatment with methionine or cysteine at physiological concentrations (100 μM) from 24-72 hpf rescued the liver size defect in *cnr1*^{-/-} and *cnr2*^{-/-} mutants at 72 hpf (Figure 2-6, A-B). In addition, treatment of methionine or cysteine protected *cnr2*^{-/-} mutants but not *cnr1*^{-/-} or wild type Tu fish from alcohol-induced steatosis (Figure 2- 6, C-D). These data demonstrate that methionine supplementation not only rescues the developmental defect of hepatocyte differentiation, but also modulates the observed metabolic injury phenotypes in *cnr2*^{-/-} mutants.

Figure 2-6 (next page). Treatment with methionine and cysteine can rescue liver development defects in cannabinoid receptor mutants

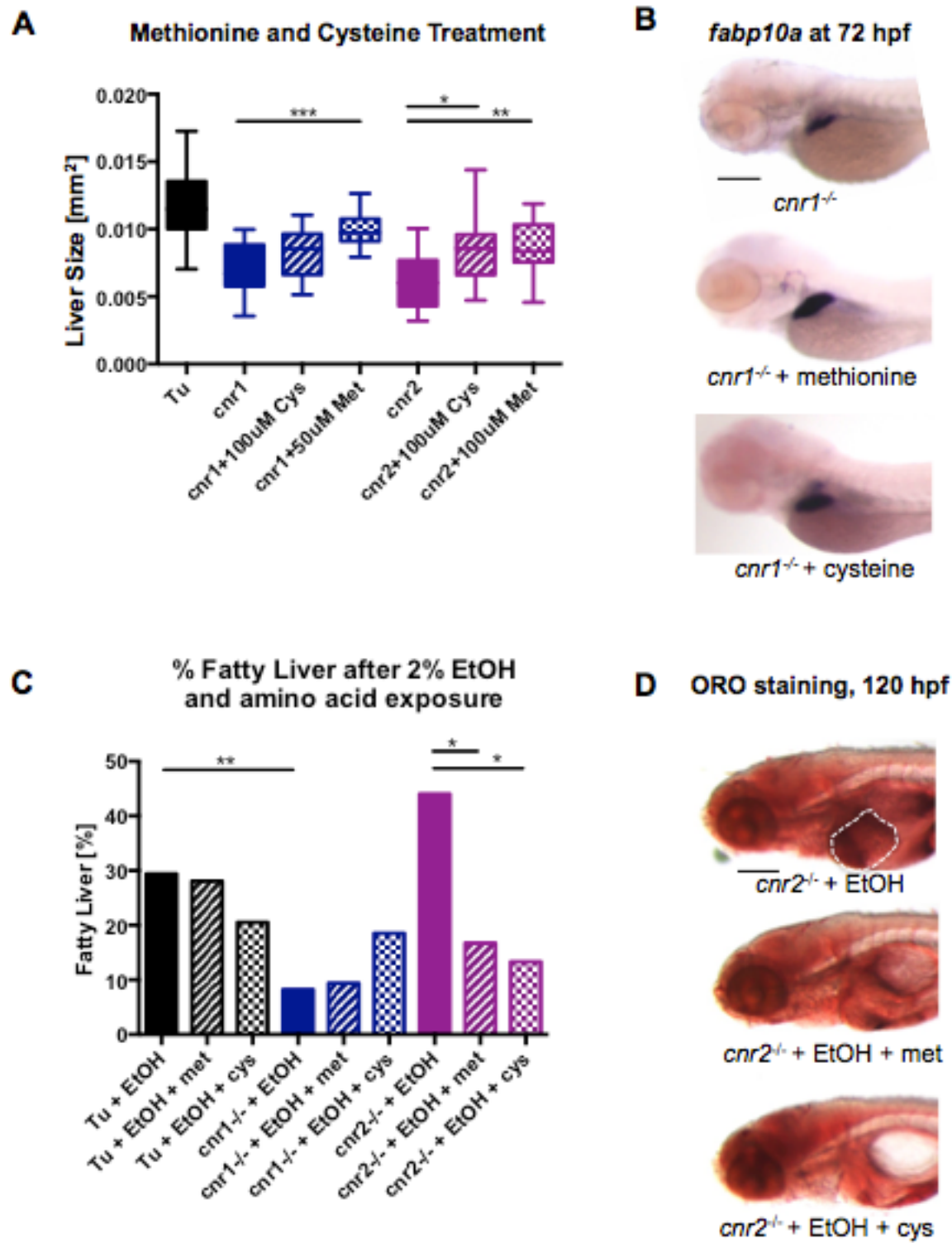
A) Box plot with liver morphometric measurements of *cnr1*^{-/-} and *cnr2*^{-/-} mutants before and after treatment with 100 μ M methionine and cysteine from 24-72 hpf. Both amino acids rescued the liver size defect in cannabinoid receptor mutants. Data are represented as mean \pm s.e.m, one-way ANOVA analysis, n > 20 samples, ***p<0.001 for *cnr1*^{-/-} compared to methionine treatment, **p<0.01 for *cnr2*^{-/-} compared to methionine treatment and *p<0.05 for *cnr2*^{-/-} compared to cysteine treatment.

B) Representative *in situ* hybridization images of *fabp10a* expression in 72 hpf embryos. Scale bar = 0.2 mm.

C) Graph showing percentage of larvae with fatty liver after ethanol-induced liver injury and with methionine or cysteine treatment. Treatment with the amino acids prevented fatty liver in *cnr2*^{-/-} mutants. Fisher's Exact test, n >30 samples, *p<0.05 and **p<0.01 comparing *cnr2*^{-/-} to methionine or cysteine treatment.

D) *cnr2*^{-/-} embryos were exposed to 2% ethanol alone or co-treated with methionine or cysteine from 96-120 hpf and scored for fatty liver based on ORO staining. Scale bar = 0.2 mm.

Figure 2-6 (continued)



SREBP mediates the effects of EC signaling

In order to characterize a potential regulator of methionine metabolism that mediates the effect of EC, we employed a candidate approach: Prior studies in *C. elegans* have shown that expression of genes involved in one-carbon metabolism is dependent on sterol regulatory element-binding proteins (SREBP) (Walker et al., 2011). We examined expression of zebrafish homologs *srebfl* and *sreb2* in *cnr1*^{-/-} and *cnr2*^{-/-} mutants at 120 hpf and adult livers, and found them to be downregulated compared to wild-type control (Figure 2-7, A). Furthermore, analysis of available expression data in *CBI* knockout mice (Mukhopadhyay et al., 2011) revealed similar downregulation of *Srebfl* and *Sreb2* (Figure 2-7, B).

To determine if SREBPs are functionally relevant for hepatocyte differentiation, we performed morpholino-mediated knockdown of *srebfl* and *sreb2*, which resulted in decreased liver formation at 72 hpf that could not be rescued by exposure to cannabinoid agonists (Figure 2-7, C-D and Figure S2-7, D-E, in appendix), indicating that *sreb* genes function downstream of cannabinoid receptors. To determine a direct relationship between cannabinoid receptors and *sreb*s, we overexpressed *srebfl* and *sreb2* in early development by injecting mRNA in one-cell stage zebrafish embryos and analyzed liver size at 72 hpf. Both *srebfl* and *sreb2* overexpression rescued liver size in *cnr1*^{-/-} and *cnr2*^{-/-} mutants (Figure 2-7, E-F), demonstrating the importance of signaling through the cannabinoid receptors via SREBPs during liver development. EC signaling therefore functions in both development and metabolism through SREBP-dependent control of one-carbon metabolism.

Figure 2-7 (next page). Endocannabinoid signaling regulates methionine metabolism via SREBPs in cannabinoid receptor mutants

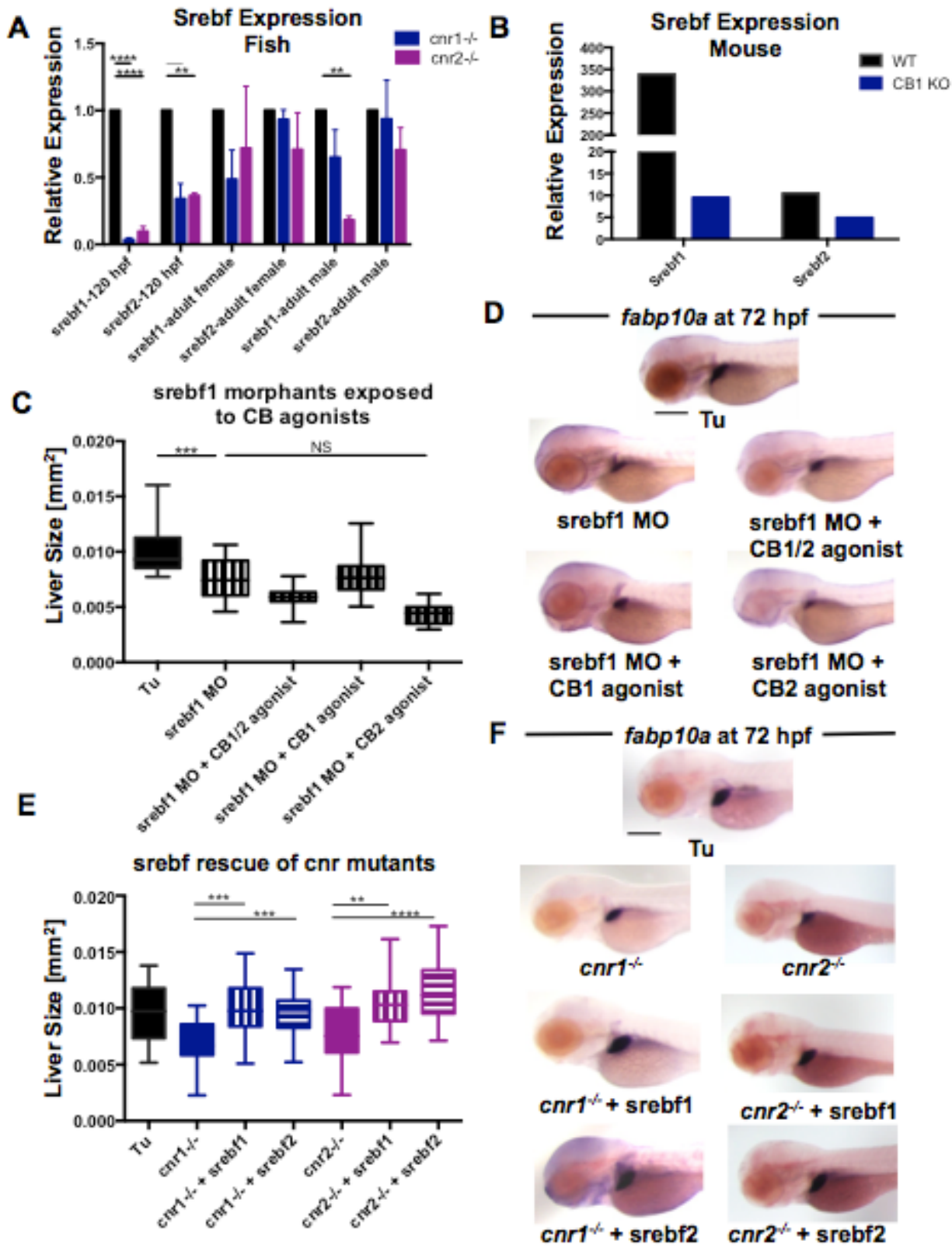
A) *srebfl* and *sreb2* levels are downregulated in *cnr1*^{-/-} and *cnr2*^{-/-} 120 hpf mutants, as measured by qPCR, but are more normalized in adult liver samples. n=3, ****p<0.0001, ***p<0.001, and **p<0.01 for expression levels of *srebfl* or *sreb2* in mutants vs. control.

B) Relative expression of *Srebfl* and *Sreb2* is decreased in CB1 knockout mice compared to control.

C) Box plot of liver morphometric measurements and D) representative *in situ* hybridization images at 72 hpf based showing reduced liver size of *srebfl* morphants and failure of the CB1/2 agonist O2545, CB1 agonist L-HCl, and CB2 agonist JWH015 treatment from 24-72 hpf to rescue liver growth when *srebfl* is knocked down. Data are represented as mean ± s.e.m with one-way ANOVA analysis, n > 20 samples, ***p<0.001 for Tu compared to *srebfl* morpholino injection. Scale bar = 0.2 mm.

E) Box plot and F) representative *in situ* hybridization images at 72 hpf based on liver morphometric measurements showing liver size of *cnr1*^{-/-} and *cnr2*^{-/-} mutants before and after overexpression of *srebfl* and *sreb2* mRNA. Both *srebfl* and *sreb2* were able to rescue the liver size defect in cannabinoid receptor mutants. Data are represented as mean ± s.e.m, one-way ANOVA analysis, n > 20, ***p<0.001 for *cnr1*^{-/-} compared to *srebfl* or *sreb2* overexpression, **p<0.01 for *cnr2*^{-/-} compared to *srebfl* overexpression and ****p<0.0001 for *cnr2*^{-/-} compared to *sreb2* overexpression. Scale bar = 0.2 mm.

Figure 2-7 (continued)



Discussion

In this study, we discover the requirement of CB1 or CB2 cannabinoid receptor activity for normal liver development: loss of CB1 or CB2 signaling impairs hepatocyte differentiation, but not earlier stages of endoderm specification. EC signaling is also important for proper hepatic physiology and metabolism during development, with defects sustained into adulthood in cannabinoid receptor mutants. Methionine metabolism, via action of SREBPs, acts downstream of the cannabinoid receptors and is functionally responsible for the observed phenotypes, as methionine supplementation or SREBP overexpression reversed liver developmental and metabolic defects in mutants. This important signaling interaction demonstrates that despite recovery of liver size, functional consequences in differentiated adult tissue still occur in cannabinoid receptor mutants.

Endocannabinoid signaling through cannabinoid receptors is required for liver development

Our results demonstrate that early metabolic dysfunction and lack of a normal hepatocyte population during hepatogenesis impedes proper liver physiology throughout development, as indicated by functional abnormalities in our larval injury models and assessment of liver physiology in larvae and adults. *cnr1* and *cnr2* have been previously found to be expressed in the zebrafish CNS, and the endocannabinoid anandamide can modulate *cnr1* expression in zebrafish (Lam et al., 2006; Martin et al., 2006; Migliarini and Carnevali, 2008; Migliarini and Carnevali, 2009; Rodriguez-Martin et al., 2007). Even though EC signaling has been implicated in promoting trophoblast cell lineage differentiation (Sun et al., 2010) and neural development (Palazuelos et al., 2011; Psychoyos et al., 2012), the role of this pathway during vertebrate endoderm and liver development has not been investigated in detail. One indication that the EC pathway may affect embryonic development was observed in CB1 knockout *ob/ob* double

mutant mice, which have growth retardation and exacerbated glucose intolerance (Li et al., 2013), thus providing additional evidence that aberrations in metabolic regulation and developmental processes are highly associated. Our data also explain why adult CB1 and CB2 knockout mice have delayed or aberrant hepatocyte proliferation compared to wild type animals after partial hepatectomy (Mukhopadhyay et al., 2011; Teixeira-Clerc et al., 2010), as the recapitulation of proliferation-promoting developmental pathways has been shown to be important during liver regeneration (Goessling et al., 2008; Nissim et al., 2014; Yin et al., 2012). CB receptors have divergent roles in mediating hepatic fat accumulation in the adult: CB1 mediates hepatic steatosis (Jeong et al., 2008; Osei-Hyiaman et al., 2008; Ravinet Trillou et al., 2004), while CB2, acting on different cell populations in the liver, inhibits lipogenesis and inflammation (Deveaux et al., 2009; Julien et al., 2005; Louvet et al., 2011b). A previous transgenic zebrafish with inducible *cnr1* expression also suggested the importance of this pathway for steatosis (Pai et al., 2013). Our data on susceptibility to ethanol and diet-induced liver injury are highly consistent with these reports. Despite the documented divergent roles of CB1 and CB2 during hepatic steatosis induction, due to differential expression on hepatocytes versus stellate cells, their common role in promoting hepatocyte proliferation during development explains the observations that these receptors are also important for proliferation of liver tissue in the regenerating liver.

Endocannabinoid signaling regulates methionine metabolism via SREBPs

SREBPs are established downstream targets of EC signaling (Jeong et al., 2008; Osei-Hyiaman et al., 2005). Although defects in EC signaling or methionine metabolism components have been independently shown to be associated with hepatic steatosis, fibrosis, or liver cancer (Huang et al., 2011; Mato et al., 2008; Pisanti et al., 2013), here we highlight a previously

unknown interaction between EC signaling, SREBP, and one-carbon metabolism. Methionine metabolism generates S-adenosyl methionine, which is required for a variety of physiological functions, including acting as a methyl donor for nucleic acid, phospholipid, and protein methylation. Methionine metabolism enzymes are conserved in zebrafish with the exception of methionine synthase, and are primarily expressed in the liver. The zebrafish mutant of S-adenosylhomocysteine hydrolase (*ahcy*), the key enzyme metabolizing S-adenosylhomocysteine into adenosine and homocysteine, is characterized by steatosis and liver degeneration (Matthews et al., 2009), while mouse knockouts for the *MAT1A*, *PEN1*, *GNMT*, *CBS*, and *MTHFR* enzymes develop steatohepatitis and hepatocellular carcinoma, potentially via decreased methylation or AMPK inhibition (Mato et al., 2008). Interestingly, a methionine-choline deficient diet has long been used as a protocol to induce features of fatty liver disease in mouse models (Best et al., 1936). Mammalian models for studying methionine metabolism enzymes also reveal the importance of this pathway for early development. Knockout of methionine synthase leads to post-implantation embryonic death, while knockout of *MTHFR* leads to smaller body size and growth retardation (Chen et al., 2001; Swanson et al., 2001). Furthermore, restriction of methionine in pregnant sheep resulted in offspring with increased adiposity and altered methylation status (Sinclair et al., 2007), and general undernutrition can cause aberrant DNA methylation in the developing embryo that perturbs adult metabolic status (Radford et al., 2014). Methionine metabolism therefore appears to be integral for proper liver function and its disruption can set the stage for lasting metabolic dysfunction. Our studies discover EC signaling as a new regulator of methionine and its essential and global functions.

SREBPs are responsible for promoting fatty acid and cholesterol uptake and synthesis and can act downstream of EC signaling in the liver, with levels that can be regulated in

zebrafish and mice by administration of anandamide (Migliarini and Carnevali, 2008; Osei-Hyiaman et al., 2005; Pai et al., 2013). In mice, modulation of dietary methionine can alter SREBP protein expression (Aissa et al., 2014), while studies in *C. elegans* show that expression of methionine metabolism genes are dependent on SREBP1 expression, and knockdown of these enzymes involved in one-carbon metabolism increases lipid accumulation (Walker et al., 2011). Furthermore, it was proposed that a feedback loop due to decreased one-carbon metabolism can activate SREBPs. Interestingly, DNA methylation of the triglyceride synthesis gene glycerol-3-phosphate acyltransferase (GPAT1) in neonatal mice decreased SREBP-1c recruitment, whereas the opposite occurred in adult mice (Ehara et al., 2012). In our studies, overexpression of *srebp1* and *srebp2* mRNA in *cnr1*^{-/-} and *cnr2*^{-/-} zebrafish embryos leads to restoration of normal liver size, providing evidence for a novel conserved signaling axis from cannabinoid receptors to methionine metabolism via SREBPs.

Modulation of EC signaling to impact energy metabolism as a therapeutic approach

Our findings allow new insights into common metabolic diseases such as hepatic steatosis, obesity, and diabetes. These diseases are all believed to have complex genetic causes, and patients with genetic variants or deficiencies of *CNR1*, *CNR2*, *fatty acid amide hydrolase (FAAH)*, the enzyme that breaks down endocannabinoids, and methionine metabolism enzymes such as *AHCY*, *PEMT*, *GNMT*, *MTHFR*, *CBS*, have a higher likelihood of developing abnormal metabolic features or liver disease (Baric et al., 2004; Coppola et al., 2014; Feng et al., 2010; Floreani et al., 2009; Mato et al., 2008; Sipe et al., 2005). Our work gives strong evidence for either the developmental defect itself or an underlying metabolic abnormality that impacts adult liver homeostasis. This offers an exciting therapeutic opportunity for early interventions that may minimize disease phenotypes later in life. Adult *cnr1*^{-/-} and *cnr2*^{-/-} mutants exhibit abnormalities

in liver and biliary morphology, and particularly *cnr2*^{-/-} mutant adult fish also have elevated serum triglyceride levels. Further study is required to determine whether rescue of the liver size defect in early development alone can prevent metabolic disease in adulthood, or whether methionine maintenance treatment in adulthood is required to provide a similar rescue. Here, we show that pathways uncovered in a zebrafish screen for novel regulators of liver development can reveal insights into developmental metabolism and adult liver homeostasis. We define a novel role for endocannabinoid signaling in promoting liver development, and discover that this pathway is essential in regulating methionine metabolism intermediates for liver homeostasis and function.

Materials and Methods

Zebrafish husbandry

Zebrafish were maintained according to standard Institutional Animal Care and Use Committee guidelines.

*Generation of *cnr1*^{-/-} and *cnr2*^{-/-} mutants*

TALE repeat arrays (<http://zifit.partners.org/ZiFiT/>) were designed to target *cnr1* and *cnr2* genes using the REAL system (Sander et al., 2011) and were cloned into TALEN vectors expressing FokI cleavage domains. TALEN mRNA pairs were injected into one-cell stage Tu embryos. Somatic mutation rates from pooled embryos were assessed (Sander et al., 2011). Injected fish were raised to adulthood and outcrossed. Founders were identified by assessing clutches for heterozygous progeny. Heterozygotes were raised to adulthood, genotyped, outcrossed for at least 3 generations, and incrossed to obtain homozygous mutants.

In situ hybridization:

In situ hybridization was conducted on embryos fixed in paraformaldehyde using standard protocols (<http://zfin.org/ZFIN/SD/ThisseProtocol.html>). Changes in *fabp10a* expression at 72 hpf were scored using ImageJ to quantify liver size in images. All other expression patterns were scored as “small,” “normal,” and “large” within a population distribution, consistent with the quantitative size distribution. The percentage altered of the total population was then calculated for each genotype/treatment group. Chi-squared test, Fisher’s exact test, and one-way ANOVA analysis were used to compare changes in the scored populations.

qPCR

RNA was extracted from pooled embryos or adult livers using Trizol and cDNA was generated using Superscript reagents (Invitrogen). qPCR was performed using SYBR Green Supermix (BioRad) and relative expression levels were calculated using the $\Delta\Delta Ct$ method. Primers are listed in Table S2-1, in appendix.

Morpholino injection

Morpholino oligonucleotides (GeneTools, LLC, Philomath, OR) were designed against *cnr1* (5’ GTGCTATCAACAACATACCTTTGTG 3’), *cnr2* (5’ GTTCCAGTTTGTCTCCATTTTCCC 3’), *sreb1* (5’ AAGTGTCGTCAAAGACAGATTCAT 3’), and *sreb2* (5’ GCGTCCATGTTTCTCCACCTTCTCT 3’). Injections were performed at the one cell stage at a concentration of 100 μ M or higher. A mismatch standard morpholino was used for control.

Oil Red O staining

Whole mount Oil Red O was conducted as previously described (Passeri et al., 2009). Embryos were scored based on presence of red lipid droplets in the liver.

Immunohistochemistry

Whole livers dissected from adult zebrafish or whole embryos were fixed, embedded in paraffin, sectioned and stained with H&E using standard protocols. H&E-stained sections were examined by Jason Hornick, a board certified pathologist. Immunohistochemistry was performed using antibodies to PCNA (Anaspec) and 2F11 (Abcam) and either FITC fluorescent secondary antibody or HRP-conjugated secondary antibody.

Analysis of fluorescent reporters in live embryos

To assess lipase activity, embryos were exposed to 0.1 µg/mL of PED6 (Farber et al., 2001) at 120 hpf for 6 hours, washed away, and then imaged using fluorescence microscopy. BODIPY-C5 fluorophore (Invitrogen) was utilized to observe lipid metabolism and digestive organ morphology *in vivo*. Embryos at 6 dpf were incubated in 6.4 µM BODIPY-C5 in 5% egg yolk solution for 4 hours, followed by confocal microscopy.

Serum lipid tests

Plasma was collected from adult zebrafish from a tail incision (Babaei et al., 2013). We analyzed plasma triglyceride and cholesterol concentrations using microplate-based enzyme activity kits (Pointe Scientific).

Polar metabolomics

Lipids were extracted from zebrafish liver samples using previously described protocols (Yuan et al., 2012). Quantitative polar metabolomics profiling was performed using AB/SCIEX 5000 QTRAP LC/MS/MS instrumentation.

Western blotting

Protein was extracted from pooled larvae populations at 120 hpf, and proteins resolved using SDS-PAGE. Presence of proteins with methylated-lysine was examined using anti-methylated lysine (Abcam) and secondary antibody conjugated with HRP (Jackson ImmunoResearch).

Chemical exposure

Cannabinoid agonists and antagonists were utilized at concentrations of 1 μ M: O-2545, Leelamine hydrochloride (L-HCl), JWH015, AM630, Rimonabant (Chemicals Cayman Chemicals, Tocris). Methionine and cysteine (Sigma) exposure occurred at 100 μ M.

mRNA injection

srebfl and *sreb2* were amplified from wild type zebrafish cDNA using the following primers: *srebfl* (5' AAGAGCATCCGAGGACAATG 3', 5' GTGTTTCAGGTGGATGTGACG 3') and *sreb2* (5' TGTGAGTGAACGAGGAGACG 3' 5' GTTATGATGCAGCGTTGGTG 3'). After PCR amplification, polyadenylated mRNA was transcribed using the mMessage mMachine transcription kit (Ambion), and injected into one cell stage zebrafish embryos at a concentration of 100 pg/nl.

Fluorescence activated cell sorting

Tg(fabp10a:GFP) embryos were manually dissociated for 10 minutes in a solution of 0.25% trypsin. Cells were strained through 35 µm nylon mesh filter and analyzed on a BD FACS Aria II flow cytometer as previously described (Goessling et al., 2008).

Endocannabinoid measurements

The tissue levels of endocannabinoids were measured by stable isotope dilution liquid chromatography/tandem mass spectrometry (LC-MS/MS) as described previously (Mukhopadhyay et al., 2011).

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Chapter 3: Functional validation of GWAS gene candidates for abnormal liver function during zebrafish liver development

Functional validation of GWAS gene candidates for abnormal liver function during zebrafish liver development

Leah Y. Liu¹, Caroline S. Fox^{2,3}, Trista E. North^{4,5}, Wolfram Goessling^{1,5,6}

¹ Genetics Division, Brigham and Women's Hospital, Harvard Medical School, Boston, MA

² National Heart, Lung, and Blood Institute's Framingham Heart Study and the Center for Population Studies, Framingham, MA

³ Division of Endocrinology, Brigham and Women's Hospital and Harvard Medical School, Boston,

⁴ Department of Pathology, Beth Israel Deaconess Hospital, Harvard Medical School

⁵ Harvard Stem Cell Institute, Cambridge, MA

⁶ Gastroenterology Division, Brigham and Women's Hospital; Gastrointestinal Cancer Center, Dana-Farber Cancer Institute, Boston, MA

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Abstract

Genome-wide association studies (GWAS) have revealed numerous associations between many phenotypes and gene candidates. Frequently, however, further elucidation of gene function has not been achieved. A recent GWAS identified 69 candidate genes associated with liver enzyme concentrations, which are clinical liver disease markers. To investigate their role in liver homeostasis, we narrowed down this list to 12 genes based on zebrafish orthology, zebrafish liver expression, and disease correlation. To assess the function of gene candidates during liver development, we assayed hepatic progenitors at 48 hours post fertilization (hpf) and hepatocytes at 72 hpf using in situ hybridization following morpholino knockdown in zebrafish embryos. Knockdown of three genes (*pnpla3*, *pklr*, and *mapk10*) decreased expression of hepatic progenitor cells, while knockdown of eight genes (*pnpla3*, *cpn1*, *trib1*, *fads2*, *slc2a2*, *pklr*, *mapk10*, and *samm50*) decreased cell-specific hepatocyte expression. We then induced liver injury in zebrafish embryos using acetaminophen exposure and observed changes in liver toxicity incidence in morphants. Prioritization of GWAS candidates and morpholino knockdown expedites the study of novel genes impacting liver development and represents a feasible method for initial assessment of candidate genes to instruct further mechanistic analyses. Our analysis can be extended to GWAS for additional disease-associated phenotypes.

Introduction

Levels of liver enzymes such as alanine aminotransferase (ALT), alkaline phosphatase, and γ -glutamyl transferase are clinical markers of liver injury, and are used to diagnose and monitor alcoholic liver disease, non-alcoholic fatty liver disease, cirrhosis, hepatitis, and drug-induced liver injury (Goessling and Friedman, 2005). Plasma concentrations of these enzymes can also be affected by heritable factors (Bathum et al., 2001), and investigating the genes influencing liver enzyme concentrations can shed light on the molecular mechanisms of liver disease. Previous genome-wide association studies (GWAS) have been conducted to uncover genetic loci associated with concentrations of plasma liver enzymes (Chambers et al., 2011; Yuan et al., 2008). Six loci were initially identified in a GWAS of 7,715 individuals (Yuan et al., 2008) and 36 additional loci were identified in a second GWAS using a larger population of 61,089 individuals, which increased the likelihood that additional genes reached statistical significance (Chambers et al., 2011). These 42 loci correspond to 69 probable candidate genes, which represent many novel associations and provide a remarkable resource for further investigation and functional insight into the disease mechanisms and molecular mediators involved in maintaining liver homeostasis. However, as with other GWAS, the size of this gene list can hinder further investigation of individual candidate genes due to the difficulty of selecting the most important or biologically relevant genes at each locus, combined with the lack of rapid and cost-effective screening methods to prioritize genes for follow-up.

Zebrafish embryos exhibit many characteristics ideal for functional validation of GWAS data, specifically rapid development, high fecundity, and population-level variation similar to humans since zebrafish are not inbred. In this study, we utilize the zebrafish embryo for functional characterization of a subset of genes found to influence liver enzyme levels in human

adults. Liver enzyme elevation in human patients is a specific measure, but reflective of a patient's susceptibility to injury and overall assessment of liver function. Previous studies revealed that pathways regulating liver homeostasis in the adult are also important for embryonic liver development, indicating the possibility of eliciting organ-relevant phenotypic changes during organogenesis (Goessling et al., 2008). Furthermore, aspects of fatty liver disease and drug-induced liver injury can be modeled in both the zebrafish adult and embryo (North et al., 2010; Passeri et al., 2009). We can also use *hhex* and *fabp10a*, genes specifying hepatic progenitors and hepatocytes, respectively, as markers of liver growth and maintenance, which are reflective of liver health and homeostasis and can be easily assessed in the zebrafish embryo. We therefore aimed to functionally validate 14 zebrafish gene isoforms representing 12 human candidate genes identified from a large GWAS, which were selected based on zebrafish conservation, expression profile, and disease correlation. We utilized morpholino knockdown of each candidate gene in the zebrafish embryo to screen for genes that impact liver development or liver injury. We found that a majority of the gene candidates were required for proper hepatocyte differentiation and a subset was also important for early specification of liver progenitors. Additionally, we discovered that susceptibility to acetaminophen-induced liver damage was altered after knockdown of three gene candidates. These results demonstrate the feasibility of using the zebrafish to characterize the function of genes identified in GWAS for liver physiology, and may be applicable to a wider variety of clinically obtained parameters.

Results

Selection of candidate genes for knockdown

Chambers et al. identified 42 genetic loci corresponding to 69 candidate genes associated with concentrations of plasma liver enzymes. Many of these genes have been previously characterized to function in lipid and carbohydrate metabolism or biliary transport, but few have been implicated in liver disease specifically (Chambers et al., 2011). To simplify this list for further study in an in vivo model system, we developed a strategy based on genomic resources, expression analysis and available functional data: we first identified the 43 genes with zebrafish orthologs, some of which have *a* and *b* isoforms (Figure 3-1). We then utilized published resources in ZFIN (www.zfin.org) to assess known zebrafish expression information to determine liver-specific expression. Subsequently, we analyzed the metabolomic, gene ontology, and KEGG pathway data from the original GWAS (Chambers et al., 2011) in combination with literature searches to select 12 genes with liver or metabolic disease correlation, but without any previously known role in liver development. We included the five genes with zebrafish orthologs that had been previously associated with elevated ALT levels, and excluded the 26 genes without zebrafish orthologs and the 31 genes without known zebrafish expression in differentiated endodermal organs such as the liver or intestine. This final list includes six genes associated with ALT elevation and/or hepatic steatosis (*MAPK10*, *CPN1*, *TRIB1*, *PNPLA3*, *SAMM50*, *MICAL3*) (Chambers et al., 2011; Sookoian and Pirola, 2011; Yuan et al., 2008). In addition, five genes are expressed in the zebrafish liver and are associated with abnormal metabolism: *SLC2A2*, encoding the glucose transporter glut2, and *PKLR* are associated with type II diabetes mellitus (Chambers et al., 2011; Hasstedt et al., 2008; Laukkanen et al., 2005); *FADS2* is associated with an altered metabolome (Chambers et al., 2011; Rzehak et al., 2010; Schaeffer, 2006); *ALDOB* is expressed

in the developing zebrafish liver and gut and implicated in carbohydrate metabolism (Chambers et al., 2011; Coffee and Tolan, 2010; Esposito et al., 2010). *MIF* is involved in lipid metabolism, immune function and fibrosis (Chambers et al., 2011; Heinrichs et al., 2011; Verschuren et al., 2009). Finally, *EFNA1* is globally expressed in the early zebrafish embryo implicated in multiple developmental signaling pathways and cancer (Chambers et al., 2011; Cui et al., 2009) (Table 3-1).

Figure 3-1 (next page). Schematic of conditions used to choose GWAS candidates for functional validation. We utilized the ZFIN database and metabolomic, disease correlation, and molecular pathway data from the liver enzyme GWAS to select 14 zebrafish genes for morpholino knockdown. We then analyzed response to acute liver injury, hepatic progenitors, and hepatocytes in morphant and control embryos to elucidate the function of candidate genes during development.

Figure 3-1 (continued)

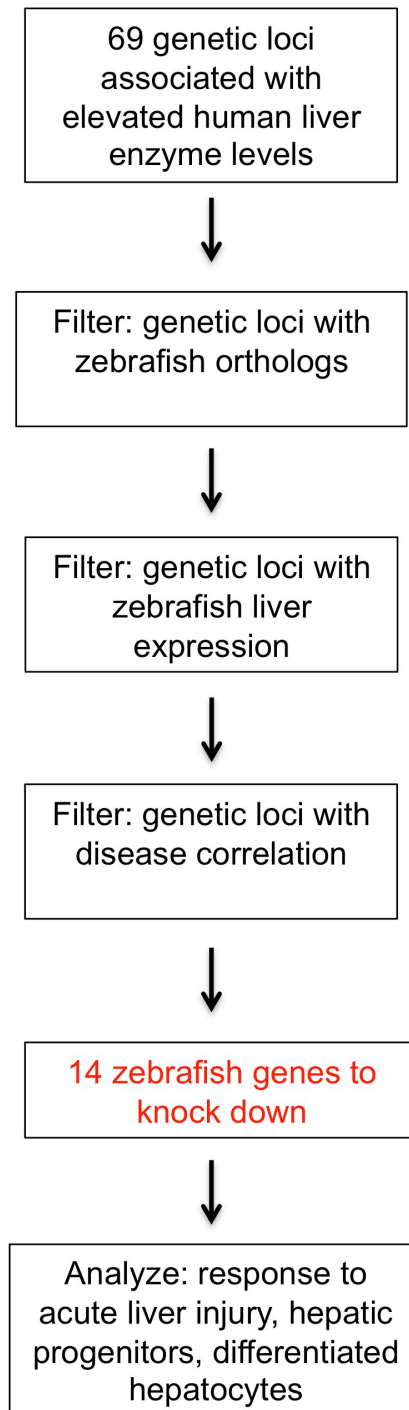


Table 3-1: 12 candidate genes for knockdown in zebrafish embryos

Gene	Full Name
<i>PNPLA3</i>	patatin-like phospholipase domain-containing protein 3
<i>CPN1</i>	carboxypeptidase N catalytic chain
<i>TRIB1</i>	tribbles homolog 1
<i>FADS2</i>	fatty acid desaturase 2
<i>MIF</i>	macrophage migration inhibitory factor
<i>SLC2A2</i>	solute carrier family 2 member 2
<i>PKLR</i>	pyruvate kinase isozymes liver and RBC
<i>MAPK10</i>	MAP kinase 10
<i>ALDOB</i>	aldolase B, fructose-bisphosphate
<i>EFNA1</i>	ephrin A1
<i>SAMM50</i>	sorting and assembly machinery component 50 homolog
<i>MICAL3</i>	microtubule-associated monooxygenase

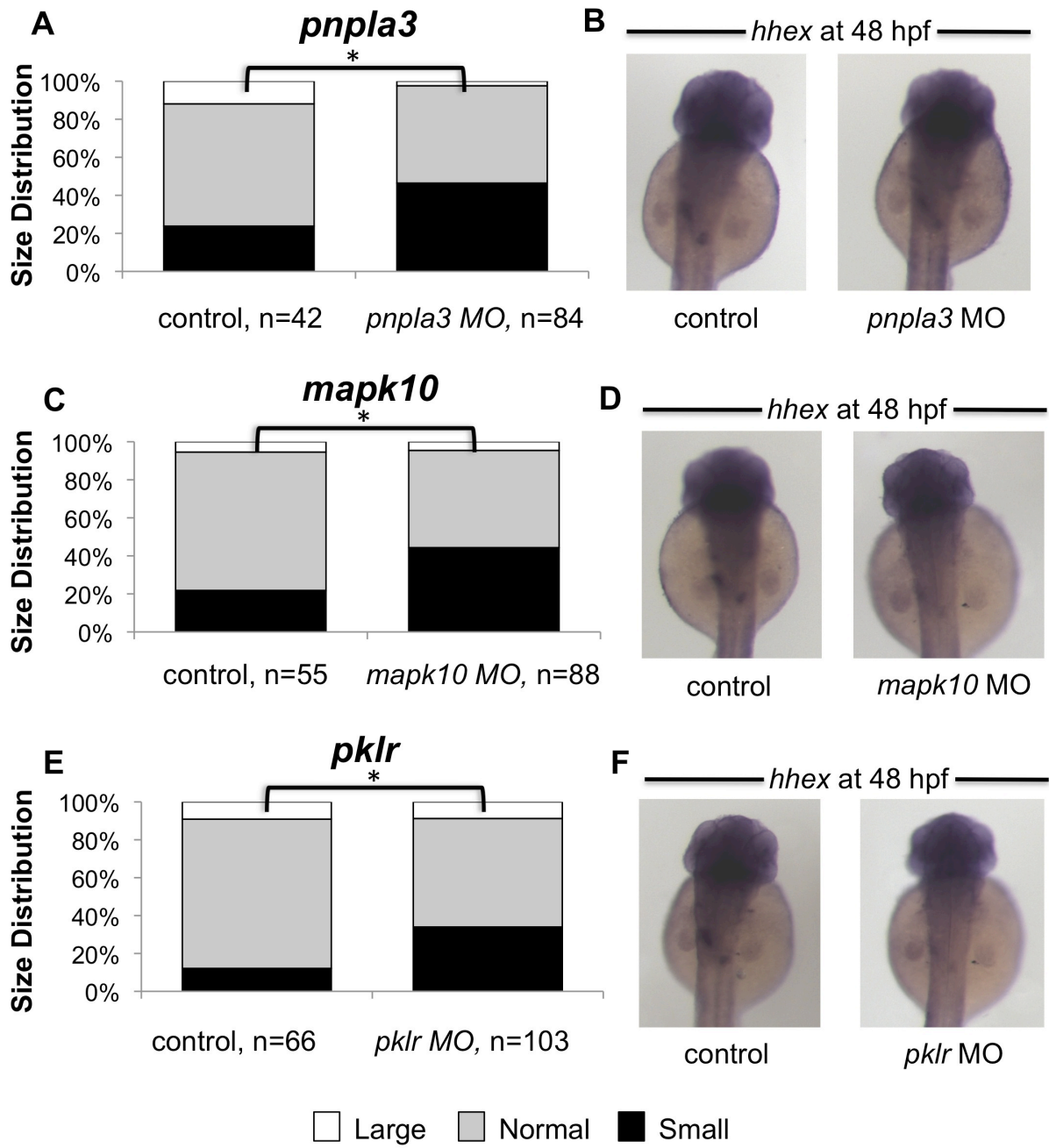
Knockdown of gene candidates impacts hepatic progenitor populations

We designed morpholino antisense oligonucleotides to target both the ATG site (Table 3-2, in appendix) and splice sites (Table 3-3, in appendix) of 14 zebrafish candidate genes, corresponding to 12 human genes found to influence plasma liver enzyme levels (*EFNA1* and *MICAL3* both have two zebrafish isoforms). We sequenced the target region of the ATG-site morpholino for each gene in both AB and Tubingen zebrafish strains to verify absence of polymorphic variation in these regions that could influence morpholino activity (Table S3-1, in appendix). Plasma liver enzyme levels are commonly used as clinical indicators of liver damage and most of the candidate genes listed in Table 3-1 are implicated in hepatic steatosis and lipid or carbohydrate metabolism. Genes governing adult liver homeostasis may also be important for liver specification and differentiation during embryonic development. In order to assess the impact of the candidate genes on hepatic development, we analyzed the expression of the hepatic progenitor marker *hhex* by in situ hybridization at 48 hours post fertilization (hpf) after morpholino injection and compared to siblings injected with a standard morpholino and uninjected controls. We scored 48 hpf embryos by designating the progenitor expression pattern as small, normal, or large. Knockdown of three genes, *pnpla3*, *mapk10*, and *pklr* using ATG-site morpholinos led to diminished *hhex* expression (Figure 3-2, B, D, F) with corresponding significant increases in the proportion of small progenitor populations within the cohort of injected embryos (Figure 3-2, A, C, E, n=42-103, Chi-squared test, $p < 0.05$). We found concordant results when using both ATG and splice-site morpholinos (Table S3-2, in appendix). These findings indicate that genes found to be associated with markers of liver injury in adults can play a role in the earliest phases of liver specification and growth.

Figure 3-2 (next page). Effect of gene knockdown on hepatic progenitor population.

Knockdown of *pnpla3*, *mapk10*, and *pklr* using ATG-site morpholinos decreased the proportion of embryos with small progenitor population in a statistically significant manner (A, C, E), as evidenced by in situ hybridization for *hhex* expression at 48 hpf (B, D, F).

Figure 3-2 (continued)



Knockdown of gene candidates impacts liver size and hepatocyte gene expression

In order to determine whether the 14 gene candidates affect liver differentiation and growth, we also examined *fabp10a* expression at 72 hpf, which is a marker of differentiated hepatocytes. Knockdown of *pnpla3*, *mapk10*, and *pklr* using ATG site morpholinos resulted in an increase of smaller livers and smaller hepatic progenitor population in the injected embryos compared to controls (Figure 3-3, A-F, n=29-173, Chi-squared test, $p < 0.05$). These observed effects could be the result of diminished hepatic progenitor numbers, or due to impaired hepatocyte differentiation and growth. In contrast, a greater number of candidate genes altered *fabp10a* expression at 72 hpf compared to *hhex* at 48 hpf: knockdown of *cpn1*, *trib1*, *fads2*, *slc2a2*, *samm50* all resulted in diminished *fabp10a* expression in a greater proportion of smaller livers compared to controls (Figure 3-3, G-P, n=29-173, $p < 0.05$). We found similar results when using both ATG and splice-site morpholinos (Table S3-3, in appendix). Five of the gene candidates tested exhibited no difference in both *hhex* or *fabp10a* expression after morpholino-knockdown (*mif*, *aldob*, *efna1a*, *efna1b*, and *mical3*, not shown). These results reveal that our selection process identified a group of gene candidates important for proper function and development of differentiated hepatocytes (Table 3-4).

Figure 3-3 (next page). Effect of gene knockdown on hepatocyte gene expression and liver size. Knockdown of 8/14 genes (*pnpla3*, *trib1*, *slc2a2*, *mapk10*, *cpn1*, *fads2*, *pklr*, and *samm50*) by ATG-site morpholino injection decreased the proportion of embryos with small progenitor population in a statistically significant manner (A, C, E, G, I, K, M, O), as evidenced by *in situ* hybridization for *fabp10a* expression at 72 hpf (B, D, F, H, J, L, N, P).

Figure 3-3 (continued)

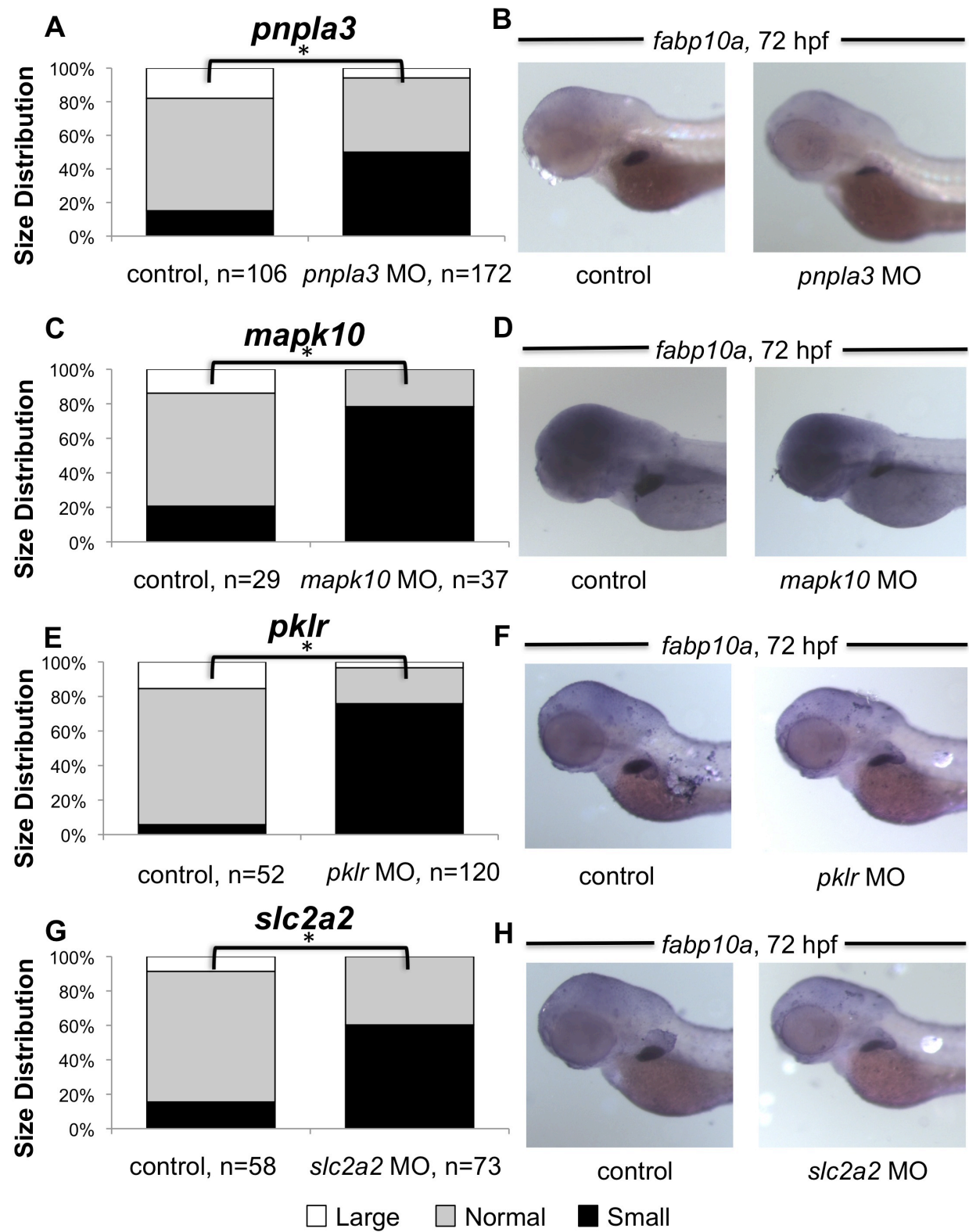


Figure 3-3 (continued)

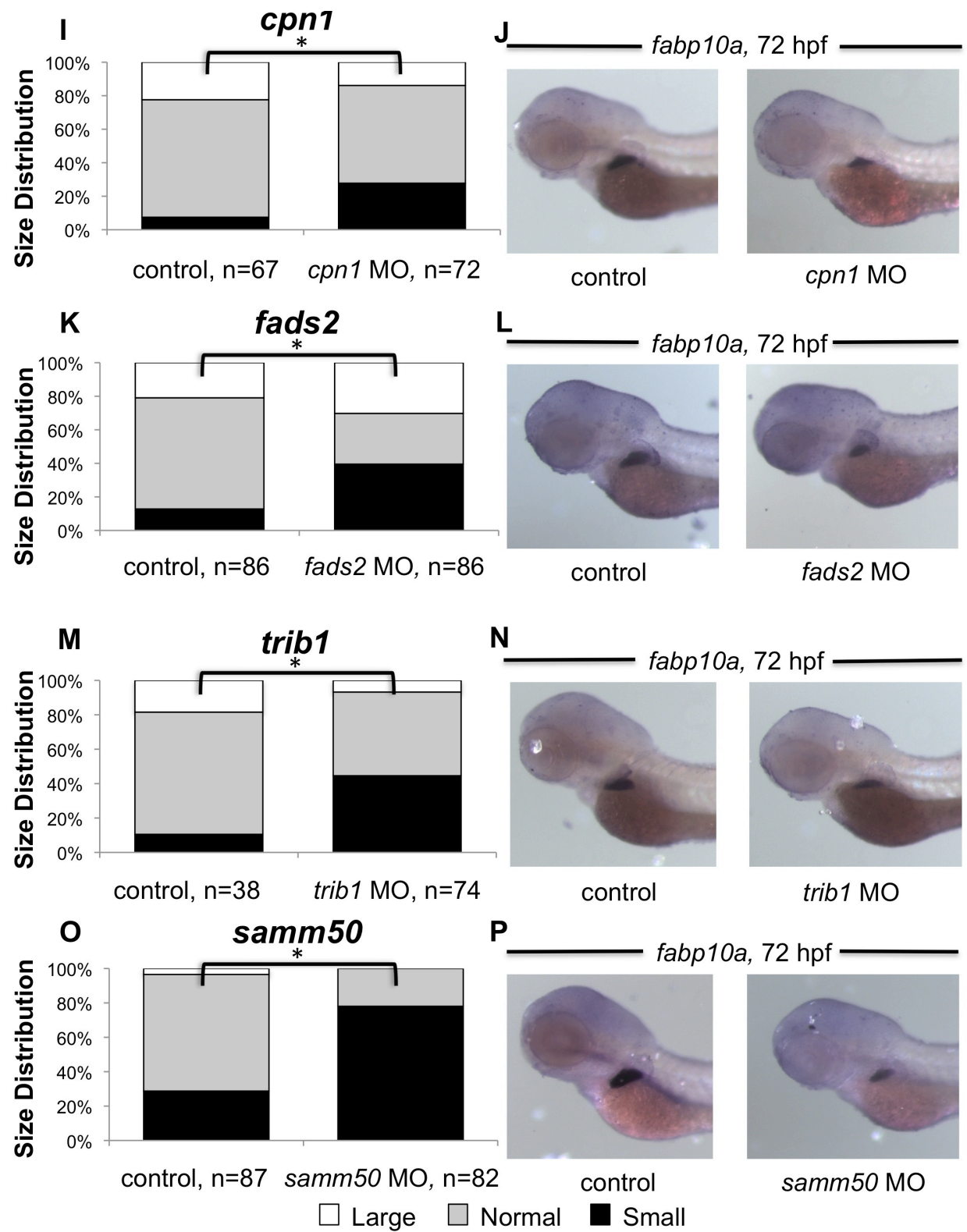


Table 3-4: Summary of liver phenotypes after ATG morpholino-knockdown of 14 zebrafish candidate genes

Gene knockdown	<i>hhex</i> expression	<i>fabp10a</i> expression	+2% EtOH (Oil Red O)	+2.5 mM APAP (<i>fabp10a</i> expression)
<i>pnpla3</i>	small	small	unchanged	reduced
<i>cpnl</i>	normal	small	unchanged	N/A
<i>trib1</i>	normal	small	unchanged	reduced
<i>fads2</i>	normal	small	unchanged	N/A
<i>mif</i>	normal	normal	unchanged	N/A
<i>slc2a2</i>	normal	small	unchanged	N/A
<i>pklr</i>	small	small	unchanged	unchanged
<i>mapk10</i>	small	small	unchanged	unchanged
<i>aldob</i>	normal	normal	unchanged	unchanged
<i>efna1a</i>	normal	normal	unchanged	N/A
<i>efna1b</i>	normal	normal	unchanged	N/A
<i>samm50</i>	normal	small	unchanged	reduced
<i>mical3a</i>	normal	normal	unchanged	unchanged
<i>mical3b</i>	normal	normal	unchanged	unchanged

Knockdown of gene candidates does not alter susceptibility to liver injury

In order to demonstrate the relevance of the developmental phenotypes for organ homeostasis, we next examined if morphant embryos show altered susceptibility to previously established models of liver injury in zebrafish embryos (North et al., 2010; Passeri et al., 2009). Exposure of 96 hpf embryos to 2% ethanol for 32 hours leads to increased neutral lipid accumulation in the liver in 50-60% of embryos as assayed by whole mount Oil Red O staining, indicative of alcoholic steatosis (Passeri et al., 2009). Morpholino knockdown of the candidate genes did not alter the susceptibility of zebrafish larvae to fatty liver following ethanol exposure when compared to uninjected or standard morpholino-injected siblings (Figure 3-4, A-B). These results indicate that loss of function of the selected candidate genes does not impair the predisposition of the embryos to respond to metabolic stress and develop hepatic steatosis.

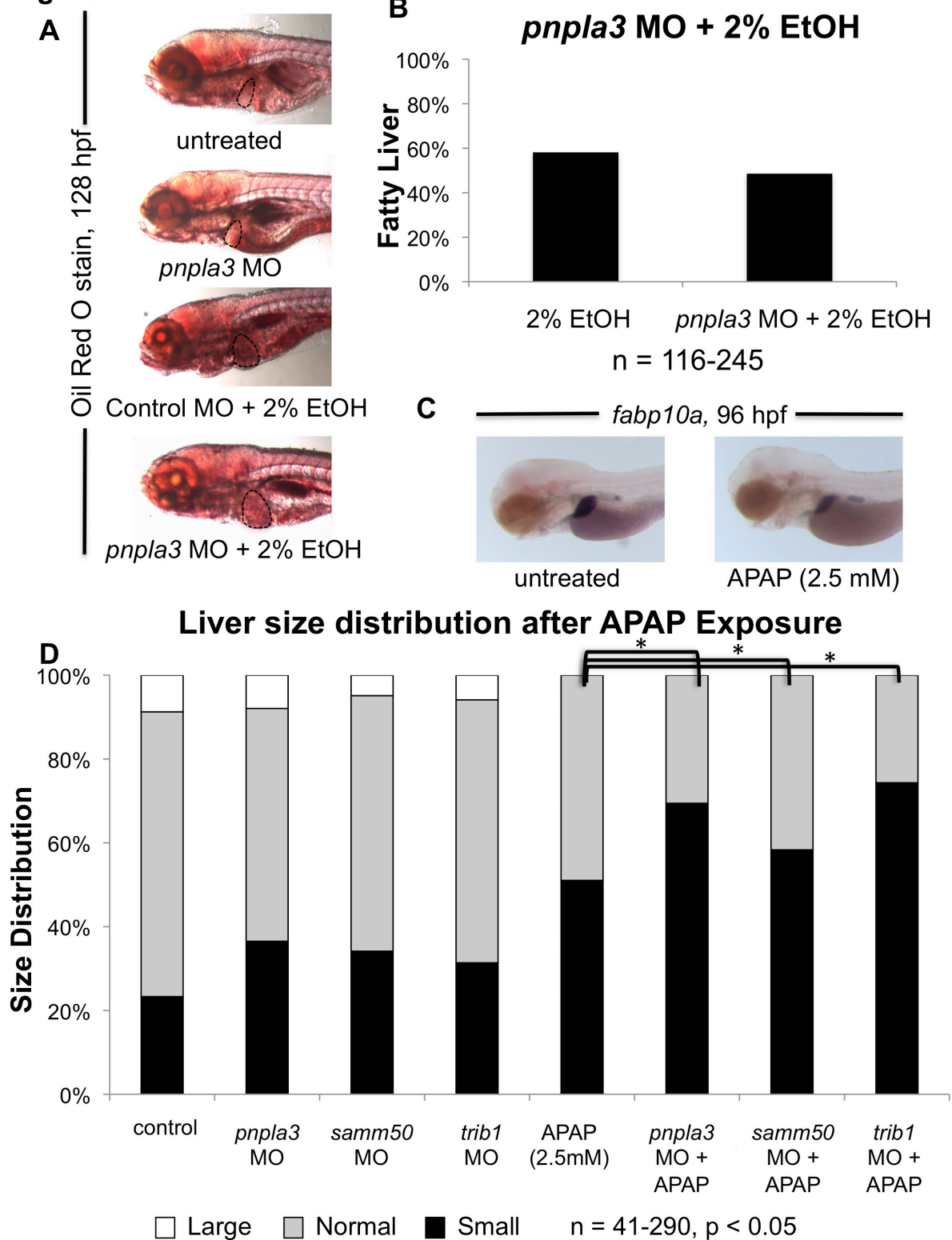
We next investigated susceptibility to toxic liver injury in a subset of gene candidates, making use of a previously developed acetaminophen (APAP) larval injury model (North et al., 2010). Exposure of 48 hpf embryos to 5 mM acetaminophen (APAP) until 96 hpf causes liver damage with smaller livers and diminished survival in the majority of embryos. We exposed morphant embryos to a lower concentration of APAP (2.5 mM) to decrease the proportion of small, damaged livers and therefore more easily observe altered susceptibility. APAP treatment of *pnpla3*, *samm50*, and *trib1* morphants resulted in a greater proportion of small livers compared to APAP treatment of controls (Figure 3-4, C-D, n=41-290, Fisher's exact test for paired controls, $p < 0.05$), whereas exposure of *pklr*, *mapk10*, and *aldob* morphants to APAP did not alter the proportion of damaged livers. We observed corresponding results when using splice-site morpholinos (Table S3-4, in appendix). However, while morphants generated using the *samm50* splice site morpholino and then exposed to APAP had reduced *fabp10a* expression

compared to controls, this was not statistically significant. This could be explained by decreased efficacy of the splice-site morpholino compared to the ATG at equal doses. Importantly, the overall observation, i.e. increased susceptibility to APAP damage, between ATG and splice-site morpholino was consistent. These results indicate that the selected genes may be important for a differential response to drug-induced acute liver injury in this model (Table 3-4).

Figure 3-4 (next page). Impact of gene knockdown on hepatic injury after ethanol or APAP treatment. Treatment of zebrafish embryos with 2% ethanol from 96-128 hpf leads to fatty droplet formation in embryonic livers, as detected by whole mount Oil Red O staining (A). Injury induction in control and ATG-site morphant embryos resulted in no statistical difference in fatty liver incidence (B). Treatment of zebrafish embryos with 2.5 mM acetaminophen (APAP) from 48-96 hpf leads to small livers compared to untreated controls, as assessed by in situ hybridization for the hepatocyte marker *fabp10a* (C). Knockdown of *pnpla3*, *samm50*, and *trib1*, followed by treatment with 2.5 mM APAP increased the proportion of small livers compared to APAP treatment alone (D).

Figure 3-4 (continued)

Figure 4.



Discussion

GWAS have been applied to a wide variety of phenotypes, ranging from chronic and infectious diseases to cancer. However, in many instances, these studies have not provided functional and mechanistic insight into the candidate genes identified. Investigating the molecular significance and function of novel genes identified by GWAS can distinguish between the true biological mediators involved in human disease pathogenesis and genes that merely correlate with disease. Furthermore, biologically relevant GWAS results can reveal novel therapeutic targets for drug design. Unfortunately, many GWAS findings lack follow-up due to the sheer number of loci and genes identified or lack of preliminary data to warrant an in-depth investigation of individual gene candidates. Here, we present an in vivo candidate-gene approach to functionally validate a subset of genes identified by a GWAS for loci influencing plasma liver enzyme levels. We narrowed down a list of 69 GWAS candidate genes in 42 loci to 14 zebrafish genes based on available zebrafish orthologs, zebrafish and/or liver gene expression pattern, and known disease correlation. We knocked down each candidate gene in zebrafish embryos using morpholinos and assessed response to liver injury, development of hepatic progenitor populations, and development of differentiated hepatocytes. Our results revealed a range of phenotypes: some morphants had unaltered livers, but the majority of morphants (8/14) exhibited smaller livers at 72 hpf compared to controls, and a subset of those morphants demonstrated a smaller progenitor population and showed enhanced susceptibility to APAP-induced liver injury. All the morphants with a decreased *hhex*-expressing domain also showed decreased *fabp10a* expression, suggesting that a smaller number of liver progenitors impacted subsequent differentiation and growth of differentiated liver cells. Due to straight-forward imaging and

analytical methods and high fecundity, the zebrafish embryo represents a cost-effective method for rapidly assessing a list of candidate genes for their biological and functional importance.

Interestingly, all genes associated with ALT concentrations resulted in smaller livers at 72 hpf after knockdown, suggesting that liver homeostasis mechanisms present in the adult liver are also functional in the developing liver and may be important for hepatocyte differentiation or development itself. Furthermore, our approach revealed the opportunity to deconvolute separate functions of genes associated with a phenotype by the same sentinel single nucleotide polymorphism (SNP): we characterized two genes that are located in the same genomic region, associated with ALT concentrations by the same sentinel SNP rs738409, *samm50* and *pnpla3*. These genes demonstrate a different phenotypic profile when knocked down during development: *samm50* morphants only exhibit reduced *fabp10a* expression, whereas *pnpla3* has diminished expression of both *hhx* and *fabp10a*. These results confirm that both genes in this locus have an important developmental requirement and may function independently at different stages of development. Remarkably, knockdown of *pnpla3* and *samm50* also enhanced susceptibility to APAP liver toxicity, as measured by reductions in *fabp10a* expression reflective of liver damage and apoptosis (North et al., 2010). This susceptibility, also found in *trib1* morphants, may be APAP specific, but may also be correlated to plasma liver enzyme elevation. For example, liver enzymes increase not only during APAP-induced liver toxicity but also in other types of metabolic or toxic liver injury, and enzyme levels can be further increased by APAP exposure (Aubert et al., 2012; Kučera et al., 2012; Majhi et al., 2011). These functional data may inform further genomic studies to identify differentiating SNPs between *pnpla3* and *samm50* that may correlate with gene function.

As with other global screening techniques, our approach may reveal false negative results: we cannot exclude an important biological function for a candidate gene even if knockdown resulted in no early liver phenotype. These genes may have redundant functions, a gain-of-function phenotype, or become active later in development or only in specific injury or disease states not tested here. For example, a specific *PNPLA3* polymorphism in humans is associated with alcoholic and nonalcoholic liver disease (Romeo et al., 2008; Tian et al., 2009), and zebrafish *pnpla3* morphants have altered susceptibility to acetaminophen but not ethanol-induced liver injury. Recently, it has been confirmed that this *PNPLA3* polymorphism confers an activating mutation (Li et al., 2012); therefore, studying zebrafish *pnpla3* morphants may not be informative with regards to the steatosis phenotype, but can still be useful for studying liver development or additional models of liver injury. The lack of statistically significant results in the ethanol injury induction assays may result from the limitations of the injury itself, which may not adequately test for the correct pathway or developmental time point important for the function of our candidate genes. Furthermore, morpholino dilution in the older, 128 hpf embryos may also account for lack of difference in the ethanol model.

However, for morphants that do exhibit a liver development phenotype, we can use this analysis as a launching point for additional in-depth studies into the molecular mechanisms and disease functions of these genes. Since these candidates were initially identified in an adult GWAS, we expected that more genes would impact differentiated hepatocytes rather than progenitors. Our assessment of hepatoblast and hepatocyte populations using in situ hybridization was semi-quantitative, enabling a rapid morphological assessment as a screening process. For those genes that are important for embryonic liver development, further quantitative and cellular analyses, such as quantitative RT-PCR for liver-specific genes, cell counts in liver-

specific reporter lines, and histological analysis, including stains for cell proliferation or cell death, will be valuable tools to provide an in-depth characterization of the morphant phenotypes. Further work may aim to distinguish the roles of individual genes in hepatocyte differentiation, proliferation, or liver function. For example, *TRIB1* has been implicated in the regulation of hepatic lipogenesis in humans, and *trib1* morpholino knockdown results in reduced *fabp10a* expression in zebrafish and increased susceptibility to APAP-induced liver toxicity.

Polymorphisms in *TRIB1* are associated with an improved lipid profile and decreased risk of myocardial infarction in humans and *Trib1* knockout mice have elevated plasma triglyceride and cholesterol levels (Burkhardt et al., 2010). We can now speculate that *trib1* may also have a role in liver development that only impacts hepatocytes since lipid metabolism may be more important in functional, differentiated liver cells, and *trib1* gene knockdown leading to elevated lipid levels may disrupt normal cell growth and proliferation, while the zebrafish embryo is still utilizing yolk as a primary source of energy. Another gene of interest is *mapk10*, which unlike *trib1*, seems to impact all stages of liver development and not only the hepatocytes when knocked down. Genetic and epigenetic alterations in human *MAPK10* have been implicated in cancers such as lymphomas and lung and liver carcinomas (Kim et al., 2005; Ying et al., 2006). Although *MAPK10* is primarily known as a pro-apoptotic factor, MAP kinase family proteins are involved in a variety of cell fate processes integral to the developing embryo and its disruption during early development may prevent the proper cell fate decision-making steps to specify and propagate the entire hepatic lineage.

The data presented here can identify genes for zebrafish knockout studies or experiments in mammalian systems. Zebrafish TILLING mutants have been identified for a handful of GWAS candidate genes, and genome editing methods such as transcription activator-like effector

nucleases (TALEN) now provide a feasible way for the zebrafish community to generate complete genetic knockouts (Bedell et al., 2012; Huang et al., 2011; Sander et al., 2011). Zebrafish mutants can be used to investigate larval and adult phenotypes and perform additional liver injury or disease-induction experiments to further elucidate the roles of GWAS candidate genes during normal liver physiology and disease.

Zebrafish embryos have previously been used as a tool for rapidly confirming and examining GWAS results in multiple organ systems such as the kidney, bone and the hematopoietic system (Gieger et al., 2011; Liu et al., 2011; Pattaro et al., 2012; Xiao et al., 2012). Here, we show that GWAS results providing a long list of gene candidates can be prioritized to characterize a feasible number of candidates for biological validation in zebrafish. We focused on 14 candidate zebrafish genes for morpholino-knockdown selected from an original list of 69 human genes associated with plasma liver enzyme concentrations, based on data from public databases and published literature. Of these 14 candidates, we identified 8 zebrafish genes required for normal embryonic liver development, three of which are also required for the normal specification of hepatic progenitors. We anticipate that these results will lead to more comprehensive analyses of individual candidate genes and their roles in liver homeostasis. Furthermore, our approach demonstrates the feasibility of utilizing large gene lists generated by GWAS for a more focused functional analysis of candidate genes. As annotation of the zebrafish genome improves and more expression data becomes available, a greater number of zebrafish orthologs from GWAS can be assessed. We expect that zebrafish will be widely utilized to confirm data from GWAS for divergent phenotypes with developmental or disease implications.

Materials and Methods

Morpholino injection

Morpholino oligonucleotides (GeneTools, LLC, Philomath, OR) were designed against the ATG start site and splice sites of 14 zebrafish genes (Tables 3-2 and 3-3). Primers were designed to sequence the morpholino target site of the ATG morpholinos in AB and Tubingen zebrafish strains to verify absence of SNPs in this region (S3-1). Each morpholino was injected into one-cell stage embryos, with uninjected embryos and embryos injected with a standard morpholino acting as controls. Injections were performed with an initial morpholino concentration of 50 μ M and increased for those morpholinos that initially caused no liver phenotype.

In situ hybridization

In situ hybridization was conducted on embryos fixed in paraformaldehyde using standard protocols (<http://zfin.org/ZFIN/Methods/ThisseProtocol.html>) and RNA probes for hematopoietically-expressed homeobox protein (*hhex*) and liver fatty acid binding protein (*fabp10a*) were used to visualize the hepatic progenitor and hepatocyte cell populations, respectively. *hhex* or *fabp10a* expression changes were assessed by scoring the expression pattern in control embryos as “small,” “normal,” and “large” within a population distribution, and compared to the size distribution of the *hhex* or *fabp10a*-expressing field in morphant embryos. The percentage altered of the total population was then calculated for each morphant group. Chi-squared test and Fisher’s exact test were used to compare changes in the scored populations.

Oil Red O staining

Whole mount Oil Red O staining of embryos was conducted as previously described (Passeri et al., 2009). Control and morphant embryos at 96 hpf were fixed in paraformaldehyde, washed with PBS and increasing concentrations of propylene glycol in PBS, and stained overnight with 0.5% Oil Red O in propylene glycol. After staining for 32 hours, embryos were washed with decreasing concentrations of propylene glycol and PBS. Embryos were scored based on presence of lipid droplets in the liver.

Translational Impact

Clinical Issue: Genome-wide association studies (GWAS) examine the relationship between gene variants and human traits, including markers of disease. Many new relationships have been uncovered using GWAS, but the confirmation and analysis of how these gene variants contribute to normal biology and disease is lacking. A recent GWAS uncovered 69 gene candidates at 42 loci associated with high levels of plasma liver enzymes, which is a marker of liver injury. Liver enzyme concentrations are used to diagnose and monitor diseases such as alcoholic and non-alcoholic fatty liver disease, hepatitis, cirrhosis, and drug-induced liver injury. The function of most of these candidate genes in liver homeostasis is unknown and methods to prioritize the genes most important to liver biology are insufficient. Determining the function of these candidates in liver biology can inform the development of new liver disease treatments.

Results: This study used zebrafish to examine the GWAS candidates that may have the greatest impact on normal liver development and function. Zebrafish give rise to hundreds of progeny at one time and zebrafish embryonic development proceeds more quickly than mammalian development, thus making this model organism an advantageous tool to rapidly

study multiple gene candidates. The authors first identified the 43 genes from the GWAS with zebrafish orthologs, and used public databases and literature to identify 12 genes most likely to have liver or metabolic disease correlation, but no known role in liver development. We knocked down these genes during zebrafish development and found that three gene candidates were necessary for liver progenitor formation, and 5 additional gene candidates were determined to be necessary for liver growth and maturation. We also found that knockdown of three gene candidates resulted in the embryos' enhanced susceptibility to toxic liver injury.

Implications and Future Directions: The data demonstrate that using zebrafish to assess knockdown of GWAS gene candidates that influence liver enzyme levels can be an effective way to prioritize genes for further study. The authors identified eight GWAS candidates that also have a role in liver development and injury, implying that factors influencing liver homeostasis in adult humans can also be important for liver maturation. Future work includes exploring the molecular mechanisms and disease functions of these genes in greater detail using zebrafish and mammalian models, mutants, and additional cellular, molecular, and physiological tools. This method to rapidly validate GWAS results can be applied to additional GWAS for other disease-associated traits.

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Chapter 4: Discussion

Summary

Nonalcoholic and alcoholic fatty liver diseases are characterized by fat accumulation in hepatocytes; however, continuous fat deposition leads to steatohepatitis and irreversible cirrhosis. These conditions are significant health concerns due to lack of treatment options and a limited understanding of disease mechanisms and associated morbidity and mortality. Liver disease pathogenesis involves not only environmental factors but also genetic and regulatory aberrations that affect embryogenesis and organogenesis. Studying novel regulators in liver development can shed light on important liver homeostatic processes that are disrupted during liver injury or chronic disease. In vertebrates, the liver is specified from the endoderm germ layer during embryogenesis. This process is conserved in zebrafish, which we use as a valuable model organism to study developmental processes and disease induction in a high-throughput manner. Zebrafish develop rapidly and externally compared to mammalian models, display remarkable fecundity, and model liver disease that can be modulated by genetic or chemical manipulation.

One approach to investigating chronic liver disease is to study novel pathways during development, followed by comparison to a similar human injury state. In Chapter 2, we discussed the role of the endocannabinoid (EC) signaling pathway, which has been previously implicated in animal models of chronic liver disease. The CB1 and CB2 receptors are expressed in the central nervous system, but also have independent effects on peripheral tissues such as the endoderm-derived gut and liver. These receptors have opposing roles during hepatic lipogenesis and fibrogenesis. Before the development of genome-editing nucleases such as TALENs or the CRISPR-Cas9 system, the zebrafish community lacked methods to generate targeted knockouts. This work is one of the first examples of zebrafish mutants generated solely for extensive phenotypic analysis and disease modeling. Using *cnr1*^{-/-} and *cnr2*^{-/-} mutants, we show that EC

signaling through CB1 and CB2 in the developing zebrafish embryo is required for normal maturation and function of hepatocytes, but not earlier milestones such as hepatic specification. We also provide evidence that methionine metabolism is an integral mediator in this process. This work gives strong evidence for either a developmental defect or an underlying metabolic abnormality that impacts adult liver homeostasis.

Another way to study regulators of liver development that become dysregulated during disease is to begin with a human data set of genes with potential disease associations, but may have unknown biological functions. Genome-wide association studies (GWAS) uncover many novel relationships, but analysis of how gene variants contribute to normal biology or disease pathogenesis is lacking. In Chapter 3, we applied a GWAS data set for elevated plasma liver enzymes, which are used to monitor liver disease progression, to show that zebrafish are an effective *in vivo* validation system for assessing a panel of genes with conserved roles in development and disease. We analyzed public databases and published literature to reduce 69 GWAS candidates to 13 zebrafish genes for assessment by morpholino knockdown. We examined morphant and control embryos for differences in susceptibility to metabolic and toxic injury as well the development of hepatic progenitor and hepatocyte populations. This approach can be applied to GWAS data sets for other traits that can be studied in a zebrafish developmental context.

Future Directions and Conclusions

The results in Chapter 2 provide intriguing insight into developmental energetics that has not been extensively studied. Initially, we sought to determine the role of EC signaling in hepatocyte differentiation and proliferation; however, we also realized the significance of this pathway for hepatic physiology and metabolism during embryo and larval stages, with defects

sustained into adulthood in cannabinoid receptor mutants. The metabolic requirements of the developing embryo have not been well characterized and how nutritional demands inform organogenesis is still an ongoing area of research.

In zebrafish, the transition from an embryo's dependence on yolk lipids to requiring food involves the confluence of several developmental and physiological milestones: the maturation and function of digestive system organs, activation of CNS and peripherally derived appetite signals, and contribution of gut microbiota. These processes have similar parallels to mammalian developmental stages *in utero* and postnatally, and current research has mostly assessed how *in utero* nutrition can impact adult health, with an emphasis on epigenetic regulation. Accordingly, our work found that disrupting a pathway important for appetite regulation, liver development, and EC signaling also impacted methionine metabolism, which is best known to generate methyl groups for cellular methylation processes such as DNA and protein methylation that are important for gene expression and regulation. Correlations between diet and environmental exposure leading to long term metabolic dysregulation have been established in mammals, and maternal diet can impact DNA methylation in offspring (Ozanne, 2014). Specifically, reducing methionine in the mammalian diet disrupts proper DNA methylation and leads to increased adiposity (Sinclair et al., 2007), and similarly, lack of proper nutrition in the embryo can lead to DNA methylation defects and aberrant adult metabolism (Radford et al., 2014).

In addition to our findings in the context of hepatogenesis, we provide new insights into different aspects of the metabolic syndrome including obesity and hepatic steatosis, which are known to have a genetic component in addition to their environmental causes. A future area of research could determine the timing and magnitude of early genetic or drug interventions that may ameliorate progressive diseases in adulthood. This could utilize adult zebrafish models of

liver disease and inducible mammalian models. Further work may also include dissecting the specific metabolic intermediates responsible for the observed effects through metabolic tracing of isotope-labeled nutrients. Additionally, cell lineage tracing at embryonic and larval stages can ascertain the specific cell populations involved, including inflammatory and immune cells not present until later developmental stages in zebrafish. We plan to conduct RNA sequencing analysis on cannabinoid receptor mutants compared to wild type animals at multiple stages of development. Comparison to polar metabolomics data can reveal metabolic derangements in other pathways, or dysregulation of metabolic enzymes. RNA sequencing can also reveal differential gene expression patterns in embryos compared to adults that could reveal how organisms grow and adapt to metabolic demands. Furthermore, dysregulation of novel pathways that have no previous association with liver development or metabolism can provide clues for additional functions of EC signaling or points of therapeutic intervention.

The work shown in Chapter 3 used morpholino injection to rapidly assess multiple genes for biological significance. Since then, advances in the genome editing field have resulted in cheaper and faster molecular biology methods to apply this technology to *in vitro* experiments and animal models alike. The efficiency of the CRISPR-Cas9 system has increased such that phenotypes can even be screened in mosaic embryos. In future studies, instead of employing morpholinos to assess a panel of genes, injection of CRISPR-Cas9 constructs may be used to screen for biological significance in newly injected embryos, which can be grown to adulthood and outcrossed to generate germline mutants. In some cases, morpholinos have failed to replicate a mutant phenotype (or lack of phenotype) seen in germline mutants (Kok et al., 2015), and the future of zebrafish research may increasingly require confirmation of phenotypes in both mutants and morphants.

Much can be gleaned from conducting screens in zebrafish. The same chemical screen that identified cannabinoid agonists as potential modifiers of liver growth also identified additional pathways involved in liver development and pathogenesis. The cannabinoid receptors were found not only to be necessary for proper liver maturation, but also for response to alcohol and nutritional-induced liver injury and as an upstream regulator of methionine metabolism. Retinoic acid, another pathway discovered by this screen, was found to be important for regulating organ laterality, while prostaglandin E2 was found to modulate the hepato-pancreas cell fate decision, and nitric oxide signaling was found to modify acetaminophen-induced liver toxicity (Cox et al., 2013; Garnaas et al., 2012; Nissim et al., 2014). These pathways were identified using the same phenotypic output, but their biological functions in liver development and liver disease proved to be diverse. The first zebrafish screens assayed mutations in early development that resulted in easily identifiable phenotypes. This particular chemical screen incorporated a fluorescent reporter to isolate a specific biological phenomenon. In the near future, variations of chemical and genetic screening may include different chemical libraries, automation, and high throughput imaging tools to survey more complex phenotypes and developmental time points.

Information from screens, human GWAS data sets, or other high throughput or candidate gene approaches can be starting points for taking advantage of the zebrafish model in developmental biology and translational research. New developments in the genome editing community are quickly being adapted to zebrafish: not only can researchers create loss of function mutations, the next frontier in genome editing includes conditional mutations, gain of function mutations, and altering gene regulation (Bedell et al., 2013; Gagnon et al., 2014; Konermann et al., 2015; Yang et al., 2013). Making observations during zebrafish development

has progressed from simple light microscopy to confocal imaging, electron microscopy, and light sheet microscopy to view biological phenomena at increasing magnification and resolution. New advances in constructing fluorescent reporters for lineage tracing, and ingestible/diffusible dyes allow for visualization of physiological processes in normal and aberrant states using live animals. All these techniques will prove useful as disease modeling in zebrafish continues to improve and manipulations are not solely restricted to the embryo but also include larval and adult stages. Studying these later developmental time points will inform interaction of multiple cell types and tissues, and the contributions of the immune system and inflammatory response that is not yet mature in the embryo.

These recent and ongoing advances will shed light on the genetic factors involved at the intersection of development and disease progression. One area of critical need in the liver disease field is generating improved models of liver fibrosis, which is ultimately the stage of chronic liver disease that destroys liver function. Mouse models of liver disease fail to recapitulate all features seen in humans, and the medical community still lacks therapeutic options that target fibrosis, restore normal hepatocyte function, and lessen the need for liver transplantation. A combination of improved zebrafish disease models, particularly in larval stages, and the development of rapid imaging or staining techniques in conjunction with a zebrafish screen for modulators of liver fibrosis could eventually lead to drug options and a better understanding of fibrosis induction and resolution.

The ultimate goal of this research is to use a combination of molecular and cellular techniques, along with animal modeling and human data to gain therapeutic insight for early interventions that may minimize adult disease phenotypes. EC signaling is only one of many pathways that function in both liver development and as a modulator of liver disease. Similarly,

elevated plasma enzyme levels are only one class of traits that are associated with liver disease progression and severity. Understanding these and related pathways, including not-yet defined regulators and how they collaborate to direct organ formation and homeostasis is key to determining the underlying molecular contributions to disease.

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Appendices

Appendix 1: Chapter 2 Supplemental Data

Figure S2-1 (next page). Cannabinoid receptor expression in the developing zebrafish

In situ hybridization images at 24, 48, and 72 hpf showing that *cnr1* and *cnr2* are expressed in the developing central nervous system, endoderm, and liver regions. Scale bar = 0.2 mm.

Figure S2-1 (continued)

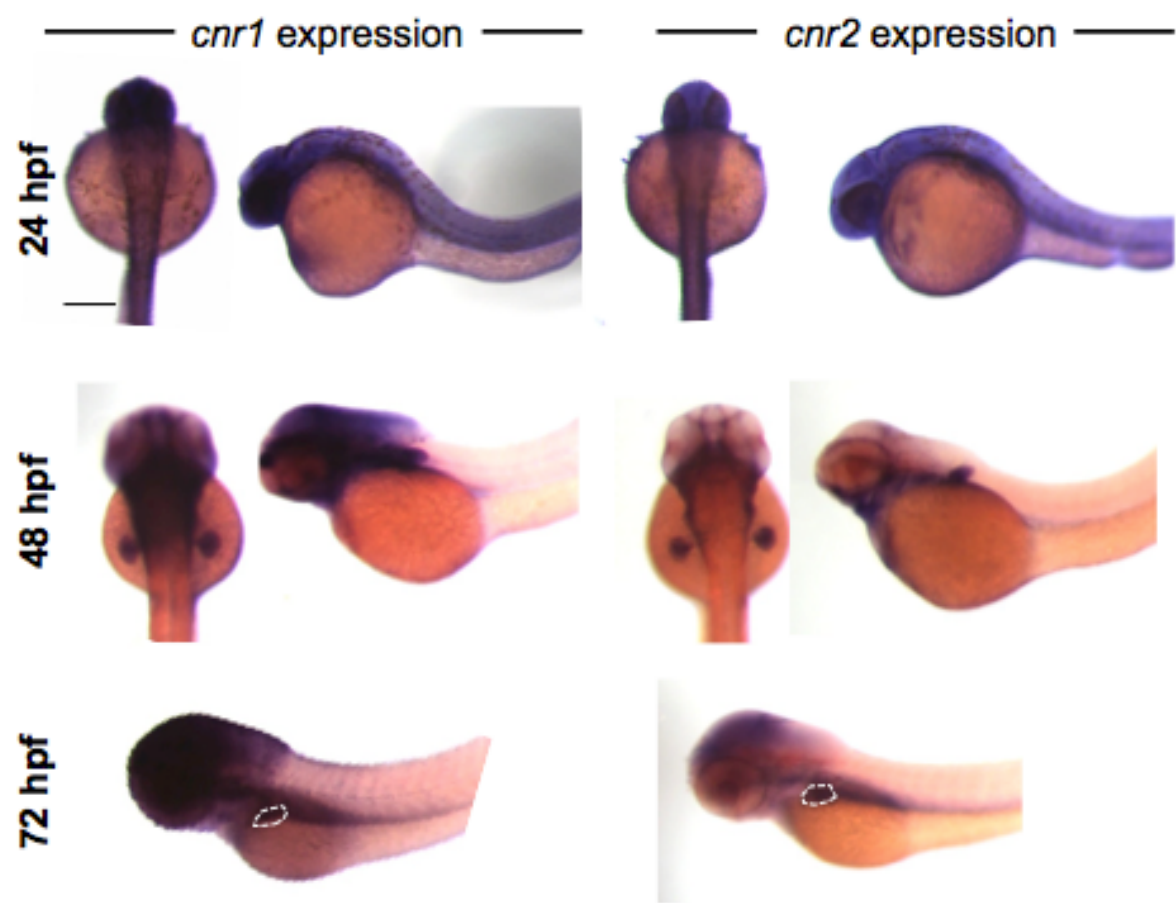


Figure S2-2 (next page). Generation of cannabinoid receptor mutants

- A) Wild-type and mutant sequence of the TALEN target region in the *cnr1* and *cnr2* genes.
- B) The small deletions shown in the DNA sequence lead to translation of an early stop codon in the first exon of both *cnr1* and *cnr2*.
- C) Box plot based on liver morphometric measurements using ImageJ showing normal liver size of *cnr1*^{+/-} and *cnr2*^{+/-} zebrafish at 72 hpf.
- D) Morpholino knockdown of *cnr1* or *cnr2* in *Tg(fabp10a:GFP)* reporter fish leads to smaller livers at 72 hpf. Scale bar = 0.2 mm.
- E) FACS quantification of *Tg(fabp10a:GFP)* embryos treated with cannabinoid agonists. % of GFP+ cells were normalized to controls.
- F) FACS quantification of *Tg(fabp10a:GFP)* embryos injected with *cnr1* and *cnr2* morpholinos. Morphants embryos at 72 hpf show an increased number of GFP positive hepatocytes at 72 hpf with agonist treatment and decreased number of GFP positive cells when *cnr1* and *cnr2* are knocked down.

Figure S2-2 (continued)

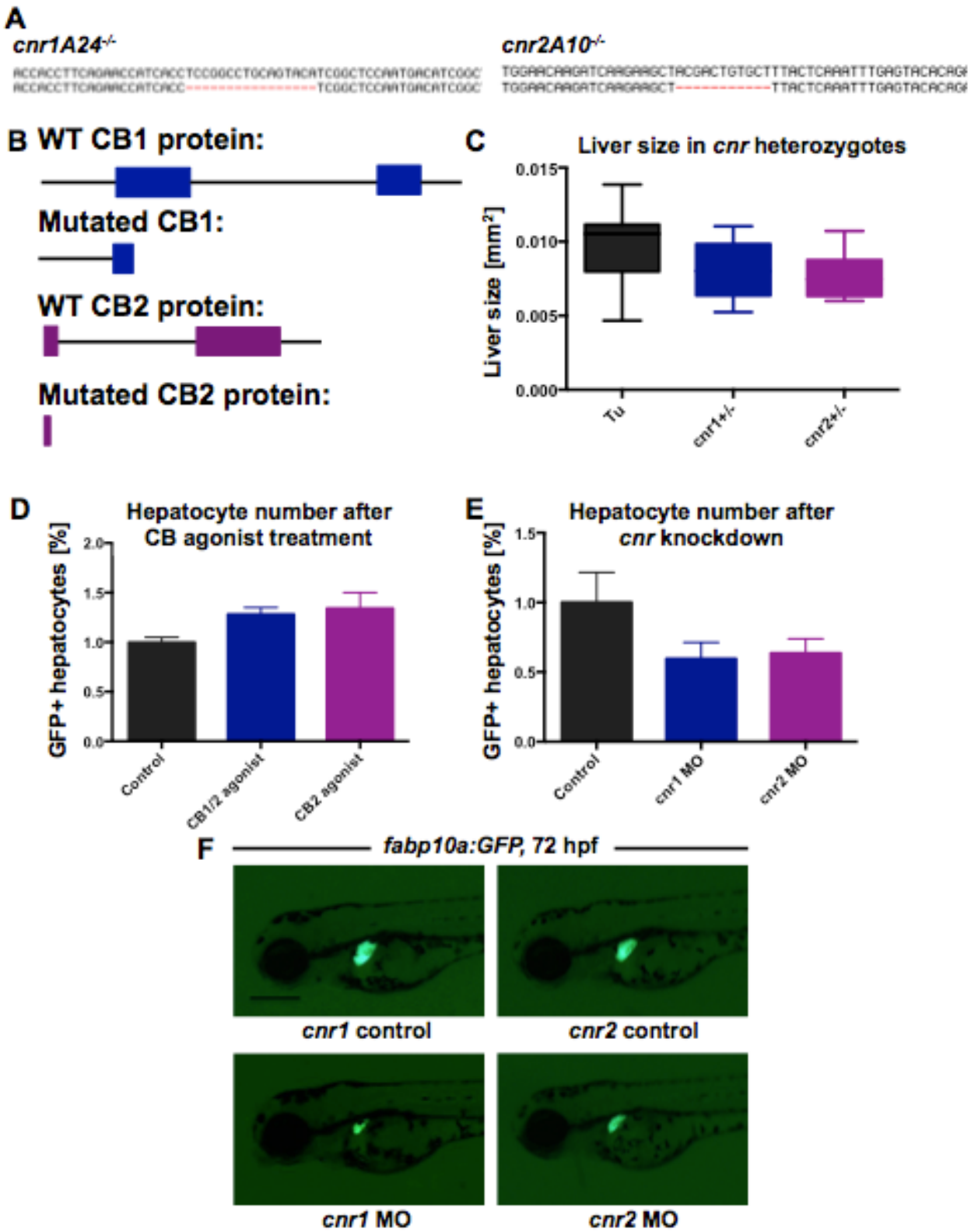


Figure S2-3 (next page). Liver phenotypes in *cnr1* and *cnr2* morphants

A) Box plot and representative *in situ* hybridization images showing liver size of *cnr1*^{-/-} and *cnr2*^{-/-} mutants at 96 hpf and

B) 120 hpf based on liver morphometric measurements using ImageJ. At 96 hpf, livers in *cnr1*^{-/-} and *cnr2*^{-/-} mutants are still significantly smaller than control, but this difference disappears by 120 hpf. Data are represented as mean ± SEM with one-way ANOVA analysis, n > 20 samples, *p<0.05 for Tu compared to and at 96 hpf only. Scale bar = 0.2 mm.

C) 120 hpf transverse sections stained for the PCNA antibody and counterstained with methyl green show decreased number of PCNA-positive cells in the cannabinoid receptor mutants compared to control. Scale bar = 0.1 mm.

Figure S2-2 (continued)

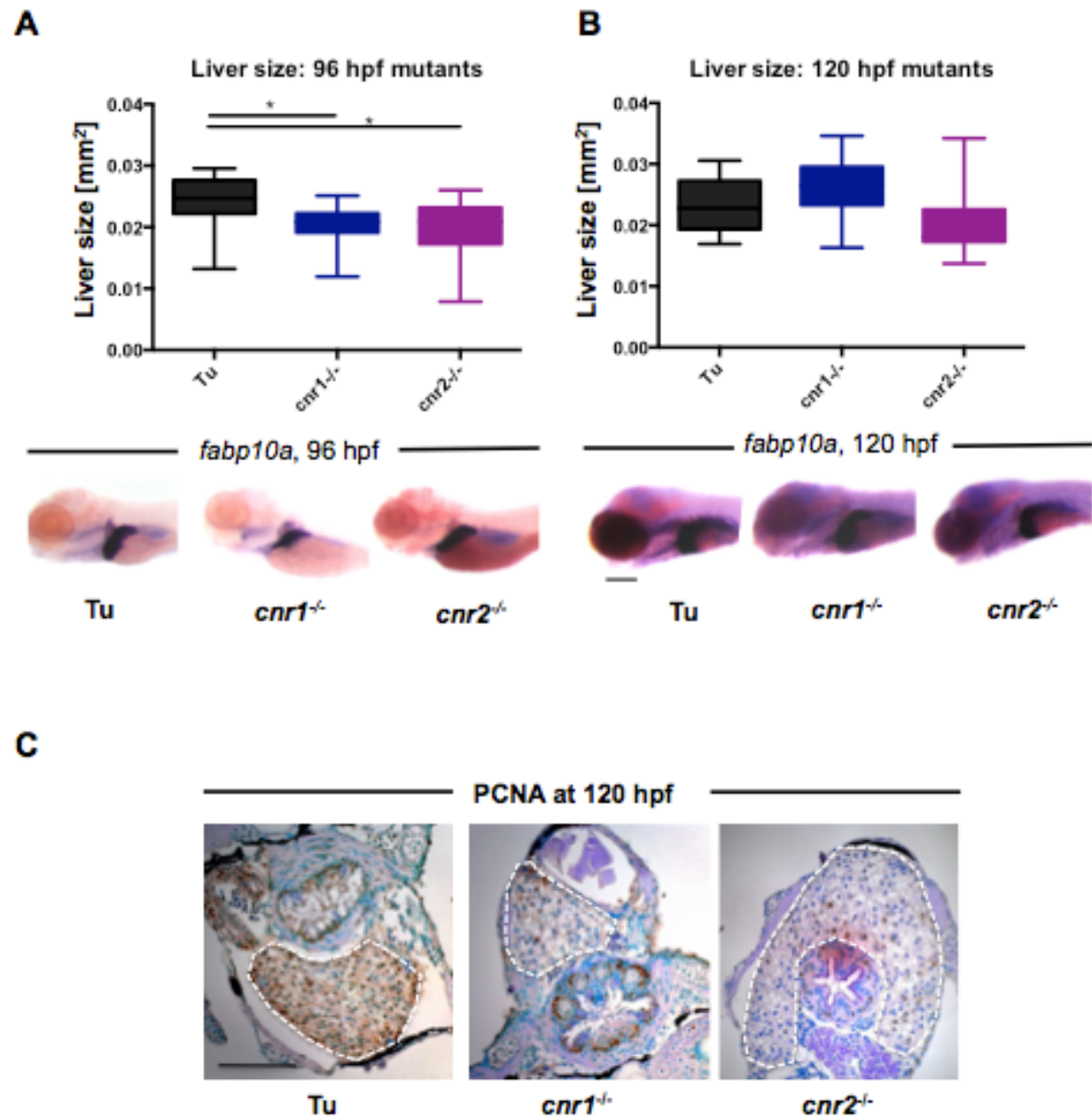


Figure S2-4 (next page). Conservation of metabolic phenotypes in zebrafish cannabinoid receptor mutants

- A) After inducing liver injury with ethanol, *cnr1*^{-/-} but not *cnr2*^{-/-} mutants at 120 hpf are protected from acquiring fatty liver as assessed by Oil Red O staining.
- B) Comparisons were made using Fisher's Exact test on >30 samples, ***p<0.001 compared to control treatment. Scale bar = 0.2 mm.
- C) *cnr1*^{-/-} mutants have decreased hepatic stellate cell activation in the ethanol-induced liver injury model based on *hand2* expression in the liver by *in situ* hybridization
- D) Fisher's Exact test, n >30, **p<0.01. Scale bar = 0.2 mm.
- E) Co-treatment with cannabinoid agonists and antagonists reveal that CB1/2 agonist increases steatosis incidence in the ethanol-induced liver injury model while the CB1 antagonist and CB2 agonist prevents steatosis induction. Fisher's Exact test, n >30, **p<0.01 and ***p<0.001.
- F) *cnr1* but not *cnr2* morphants are protected from induction of steatosis in the ethanol-induced liver injury model. Fisher's Exact test, n >30, **p<0.01.

Figure S2-4 (continued)

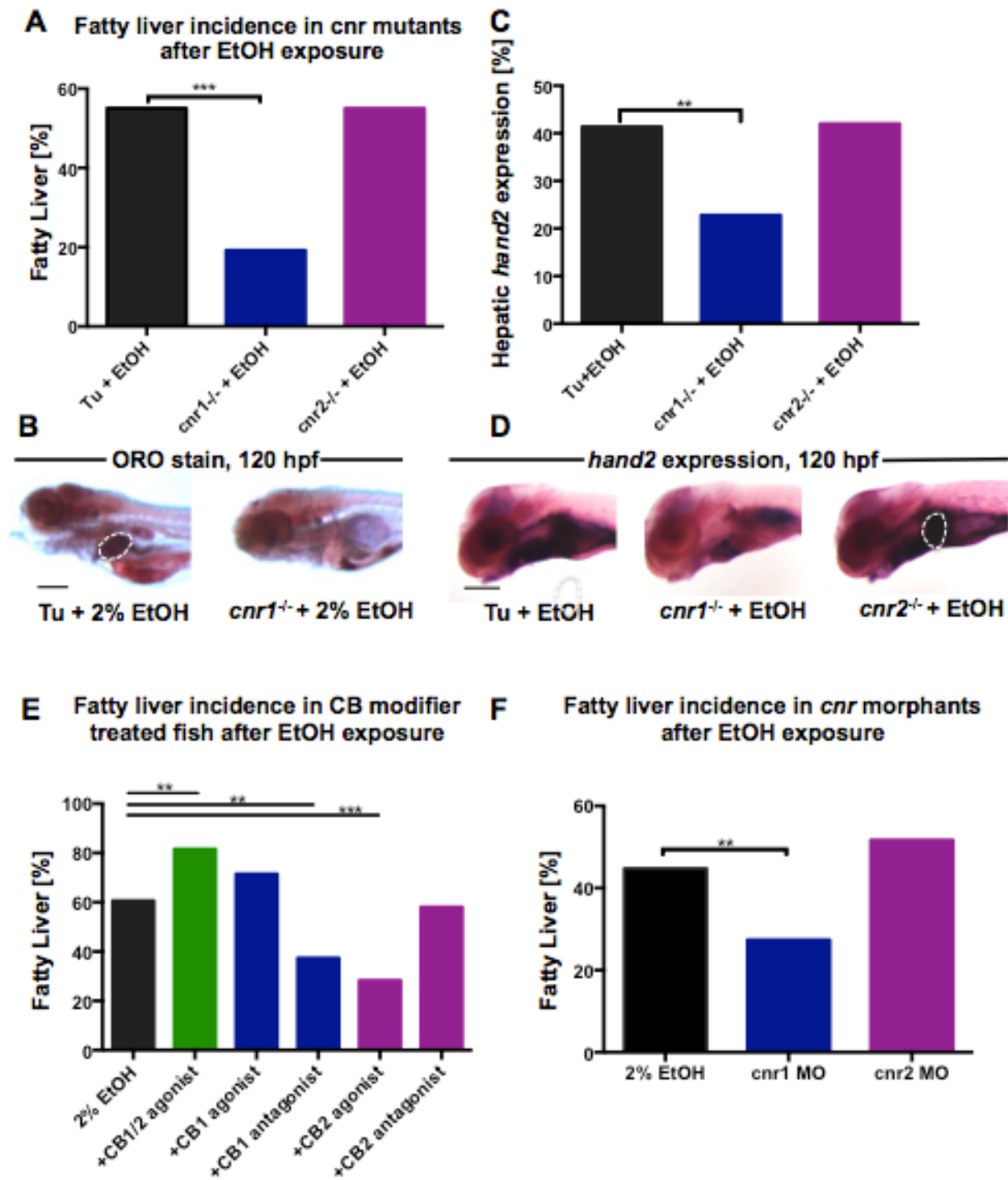


Figure S2-5 (next page). *cnr1* morphants and treatment with CB1 antagonist protects from hepatic steatosis and *hand2* induction

A) *cnr1*^{-/-} but not *cnr2*^{-/-} mutants fed a high fat egg yolk diet from 96-120 hpf are also protected from fatty liver, as indicated by Oil Red O staining.

B) Fisher's Exact test, n >30 samples, **p<0.01 vs. controls. Scale bar = 0.2 mm.

C) Co-treatment with cannabinoid agonists and antagonists reveal that CB1 antagonist and CB2 agonist prevents steatosis induction in a high fat diet egg yolk feeding model. Fisher's Exact test, n >30, *p<0.05 and ***p<0.001 vs. controls.

Figure S2-5 (continued)

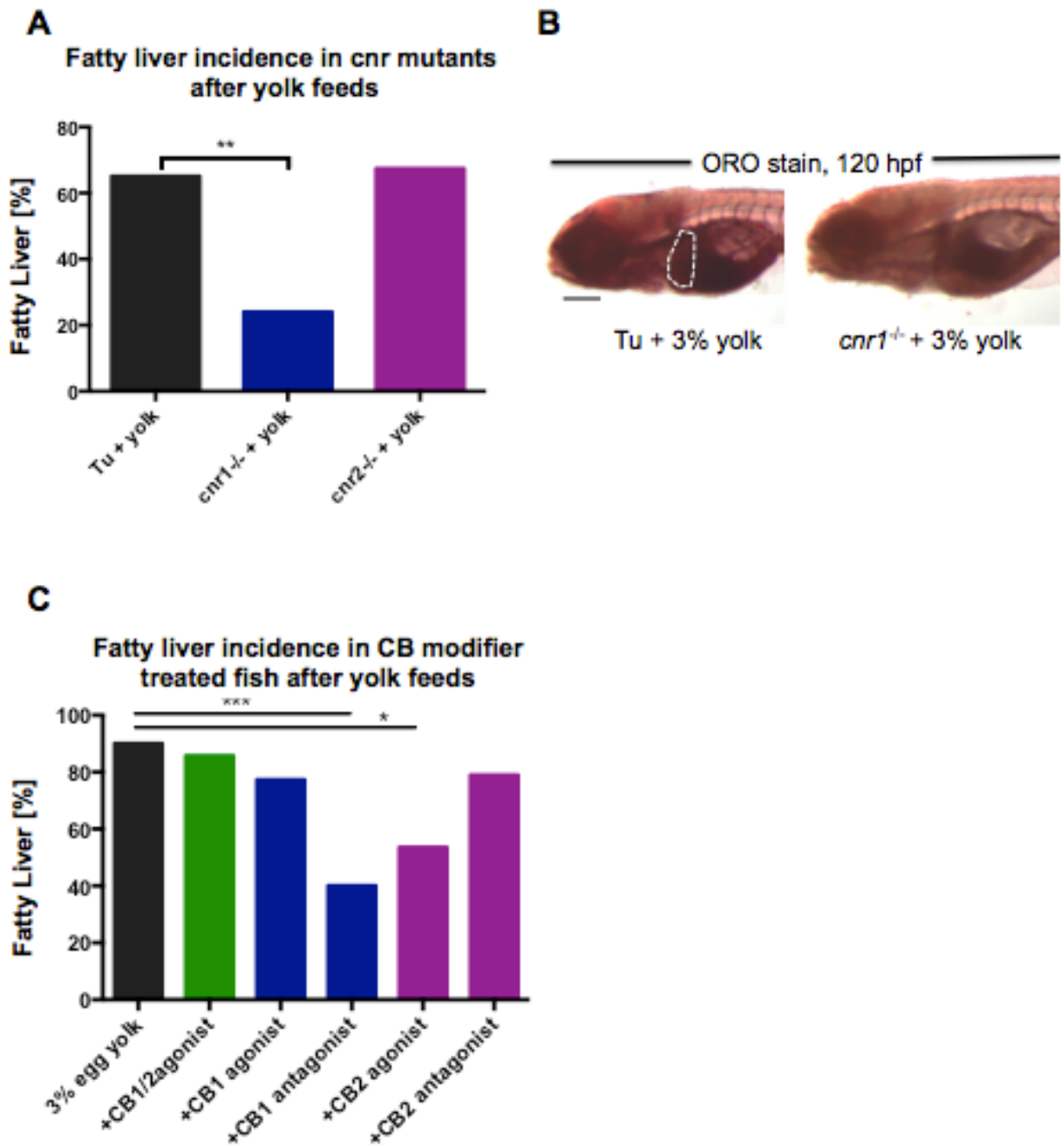


Figure S2-6 (next page). Measurement of endocannabinoid content in cannabinoid receptor mutants

A) Anandamide (AEA) and

B) Arachidonylglycerol (1-AG and 2-AG) content in adult liver samples were measured by liquid chromatography/tandem mass spectrometry, revealing no statistically significant differences between control and mutant samples. Data are represented as mean \pm SEM, n=2.

C) Liver mass to body mass ratio measurements in female and D) male adult *cnr1*^{-/-} and *cnr2*^{-/-} mutants show no difference in liver size between cannabinoid receptor mutants and controls by adulthood.

Figure S2-6 (continued)

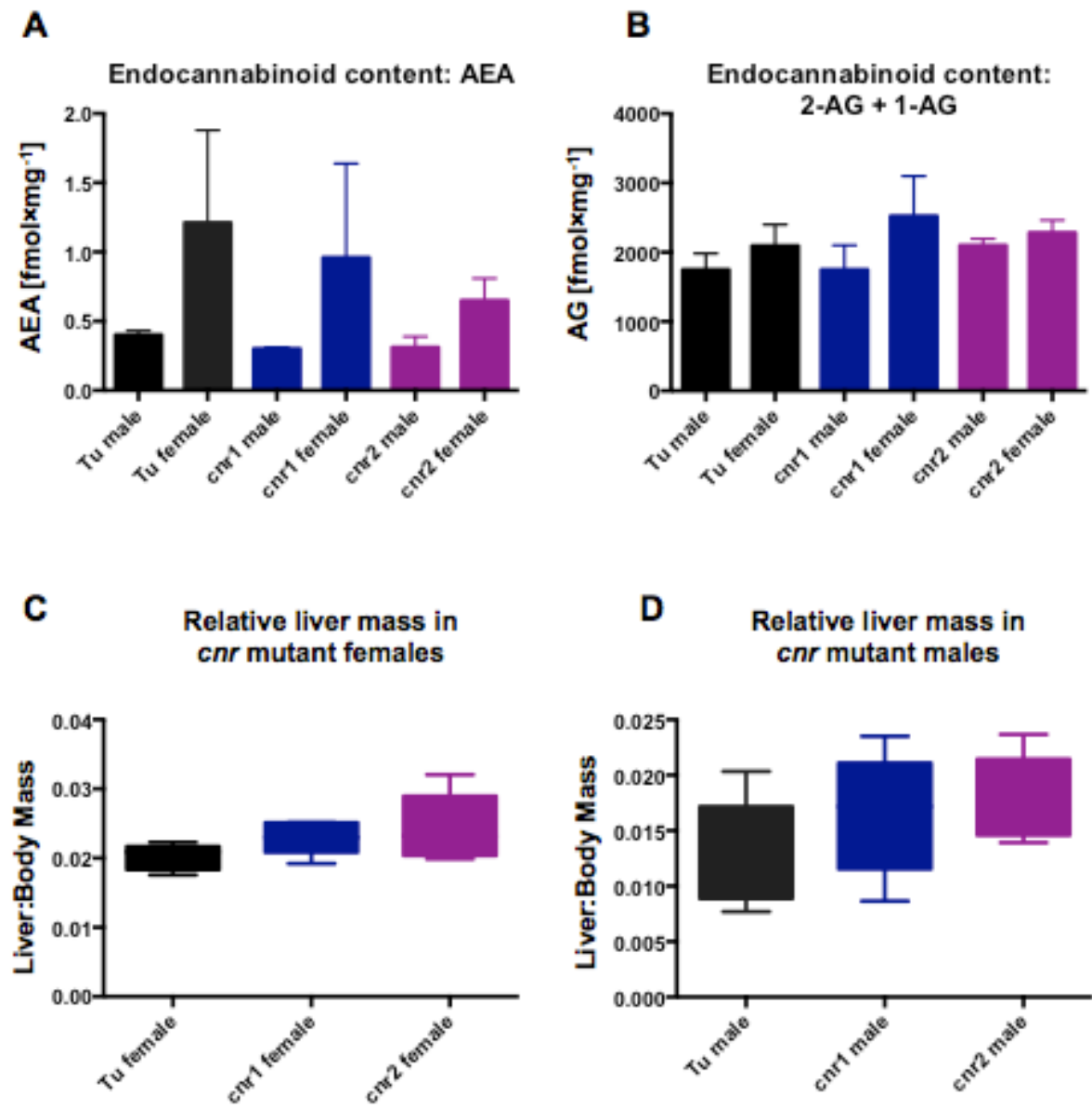


Figure S2-7 (next page). Relative methionine metabolite levels after polar metabolomics analysis of adult livers

A) Heat maps show metabolites that are most dysregulated between wild type and *cnr1*^{-/-} or *cnr2*^{-/-} mutants with $p < 0.05$.

B) Polar metabolomics analysis reveals relative levels of methionine pathway metabolites from male and

C) female *cnr1*^{-/-} or *cnr2*^{-/-} adult livers. Data are represented as mean \pm SEM, $n=3$.

D) Box plot and

E) representative *in situ* hybridization images at 72 hpf based on liver morphometric measurements showing reduced liver size of *sreb2* morphants and failure of the CB1/2 agonist O2545, CB1 agonist L-HCl, and CB2 agonist JWH015 treatment from 24-72 hpf to rescue liver growth when *sreb2* is knocked down. Data are represented as mean \pm SEM with one-way ANOVA analysis, $n > 20$ samples, **** $p < 0.0001$ for Tu compared *sreb2* morpholino injection.

Figure S2-7 (continued)

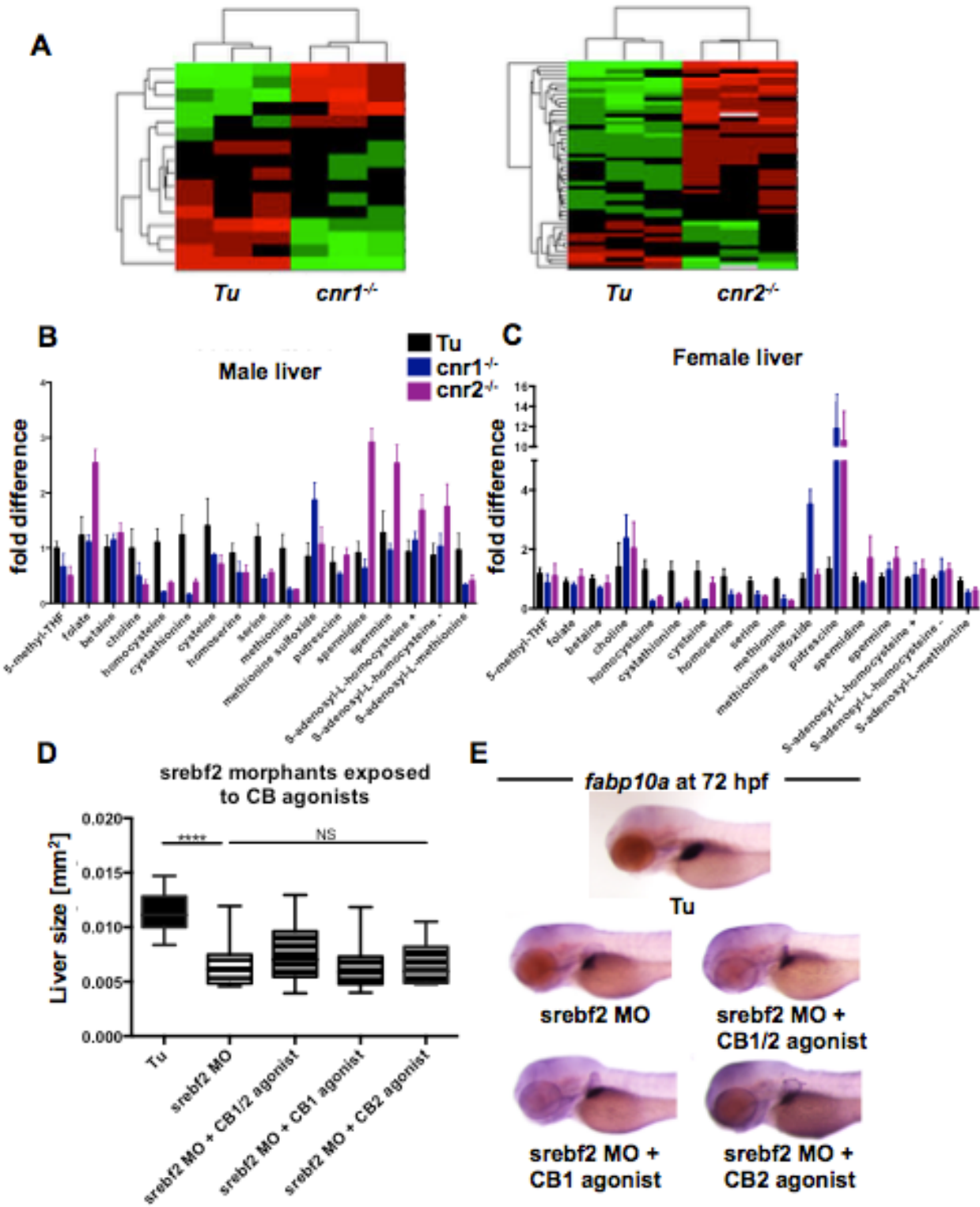


Table S2-1: qPCR primer sequences

Gene	Forward Primer	Reverse Primer
<i>fabp10a</i>	GATGGAGGAAAGCTGGTCTG	TCCTGATCATGGTGGTTCCT
<i>ahcy</i>	ACCAGACAGCACAAACGTCAA	AGACCCGGCATCTCATTCT
<i>mthfr</i>	GTCTGGAGCGGTCAATCTTATC	CGGGATGCCAAGTGATGT
<i>bhmt</i>	GCATGGAGTTACACCTGGAGA	CACAGTCTTCACACAGGTCAGG
<i>srm</i>	CGAAGTTCCAGGATGTGATG	GGTGGCAGCAGAGAGGTAGA
<i>srebf1</i>	GTCTGTCTGGCTTCACCAATC	GAGAGTCGGCCTTAATGAACTG
<i>srebf2</i>	ACCATACAGACGCTCTCCACA	GGTTTGGTGGTCAGAAGCAG

Appendix 2: Chapter 2 Ongoing experiments

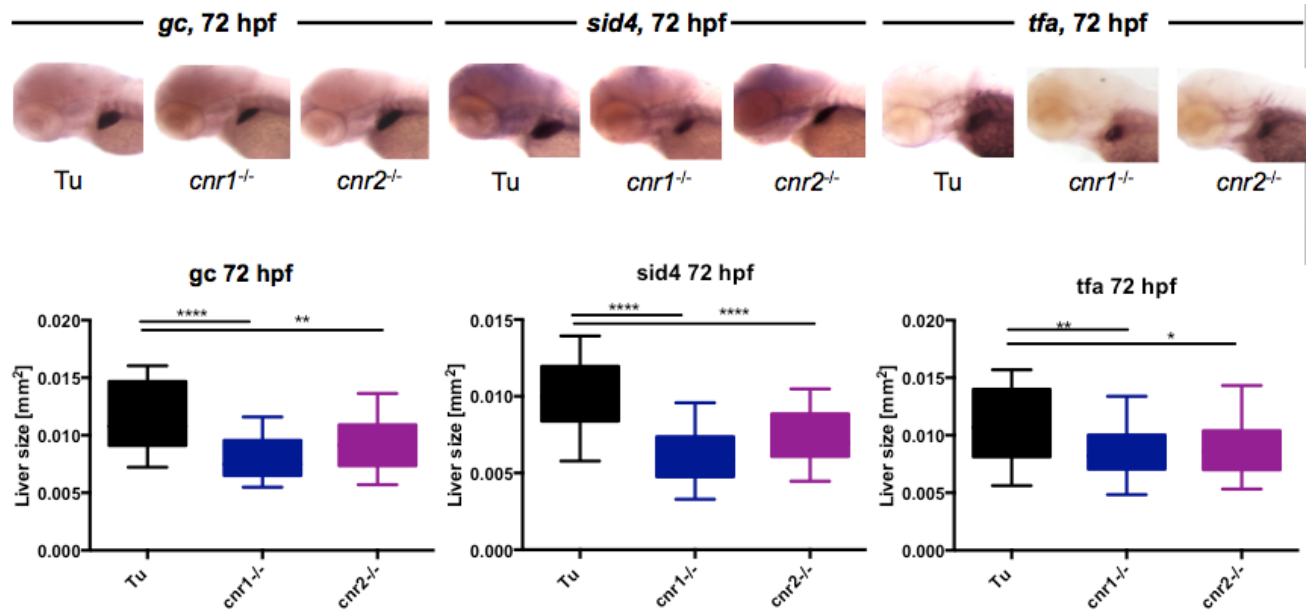


Figure S2-8. Expression of additional hepatocyte markers in *cnr1*^{-/-} and *cnr2*^{-/-} mutants

We assessed the expression of *group-specific component* (vitamin D binding protein) (*gc*), *secreted immunoglobulin domain 4* (*sid4*), and *transferrin* (*tfa*), which are additional *in situ* hybridization markers for differentiated hepatocytes. We observed decreased expression of these markers in 72 hpf *cnr1*^{-/-} and *cnr2*^{-/-} mutants compared to control wild type embryos, confirming smaller liver size and excluding a dependence on *fabp10a* expression when cannabinoid receptors are knocked out in these mutants.

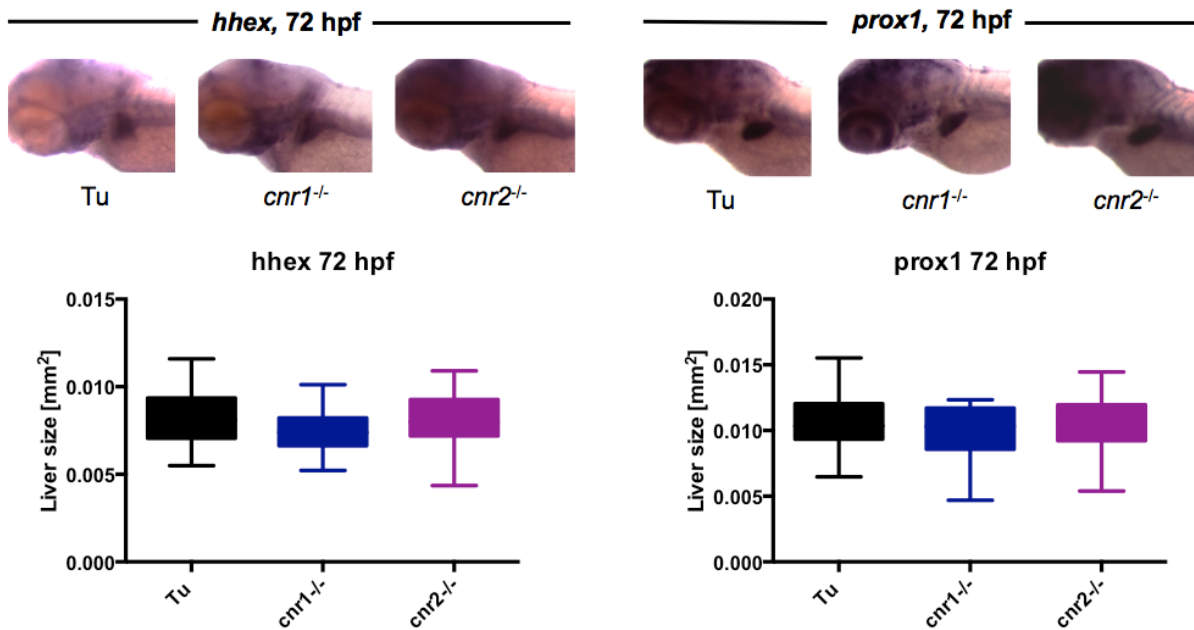


Figure S2-9. Hepatic progenitors remain unchanged in *cnr1*^{-/-} and *cnr2*^{-/-} mutants at 72 hpf
 We assessed the expression of *hhcx* and *prox1*, which are *in situ* hybridization markers for hepatic progenitors. We observed no difference in expression pattern when comparing wild type and mutant embryos at 72 hpf. Along with the results in Figure S2-8, these data suggest that disruption of signaling through cannabinoid receptors impacts hepatic differentiation.

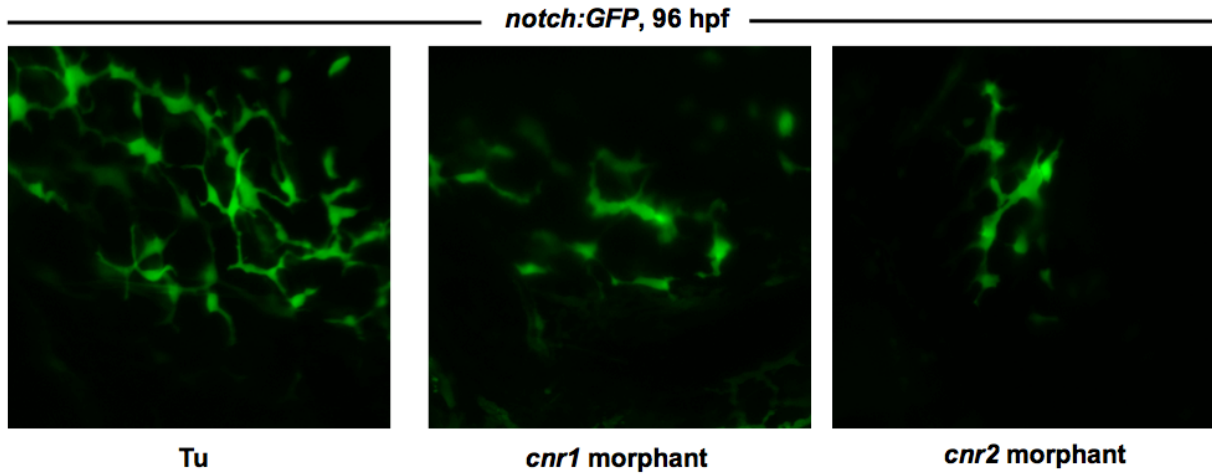


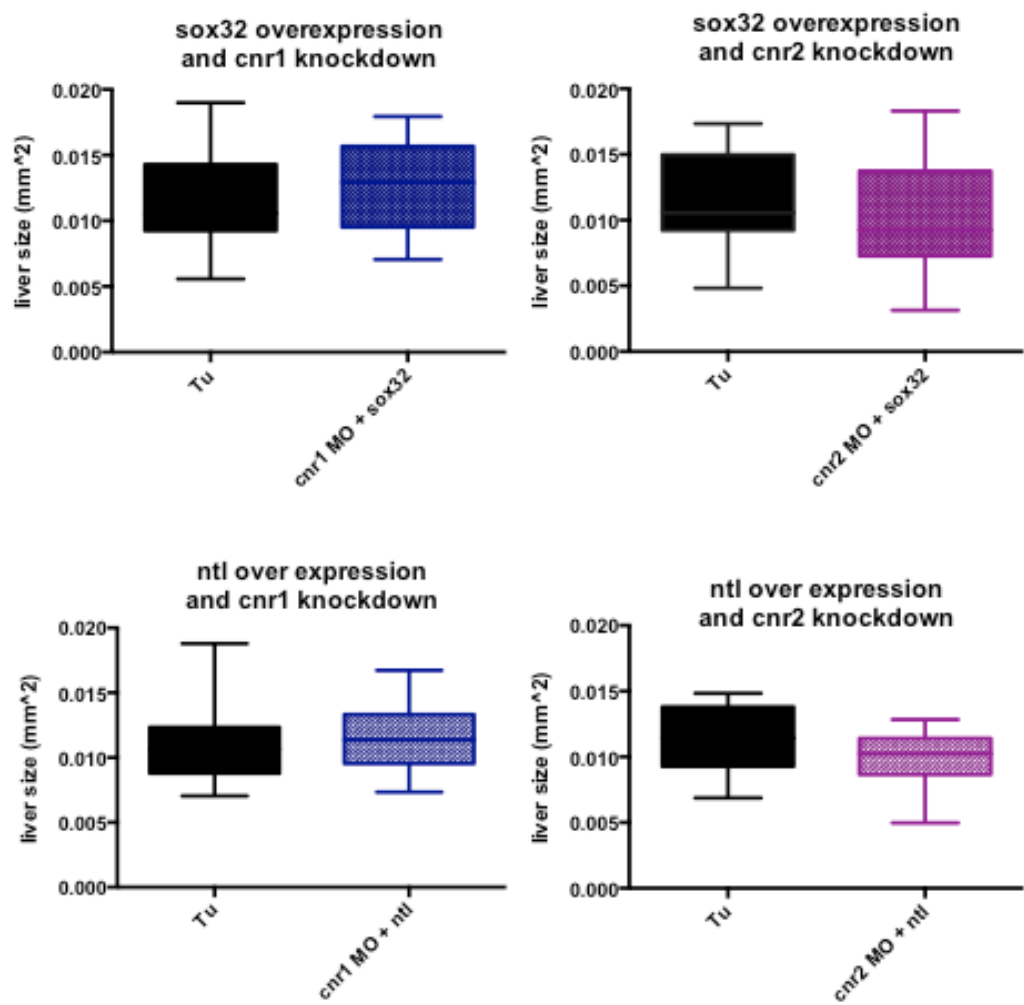
Figure S2-10. Knockdown of *cnr1* and *cnr2* impedes proper biliary tree formation

We injected *cnr1* or *cnr2* morpholino in the *Tg(notch:GFP)* transgenic reporter, which expresses GFP in the biliary tree. At 96 hpf, we imaged the liver in morphant embryos by confocal microscopy and observed smaller liver size and decreased biliary branching and formation compared to control. Representative images are shown.

Figure S2-11 (next page). Investigating endocannabinoid signaling and cell autonomy

We utilized a previously established system for driving morpholino knockdown exclusively in endodermally derived cell populations: *sox32* is necessary and sufficient for endoderm formation in zebrafish, and overexpression of *sox32* while co-injecting a morpholino of interest in 32-cell stage embryos restricts morpholino action to *sox32*-derived cell populations. When we co-injected *sox32* mRNA with *cnr1* or *cnr2* morpholino, we did not see any changes in liver size compared to control, suggesting that signals from outside the endoderm were responsible for the observed phenotypes. Subsequently, we conducted a similar experiment in the mesoderm using overexpression of the mesoderm marker *ntl* (Kristen Alexa, unpublished results); however, this also did not result in any changes in liver size. Therefore, it may be possible that signals from multiple germ layers are necessary for endocannabinoid action during liver development.

Figure S2-11 (continued)



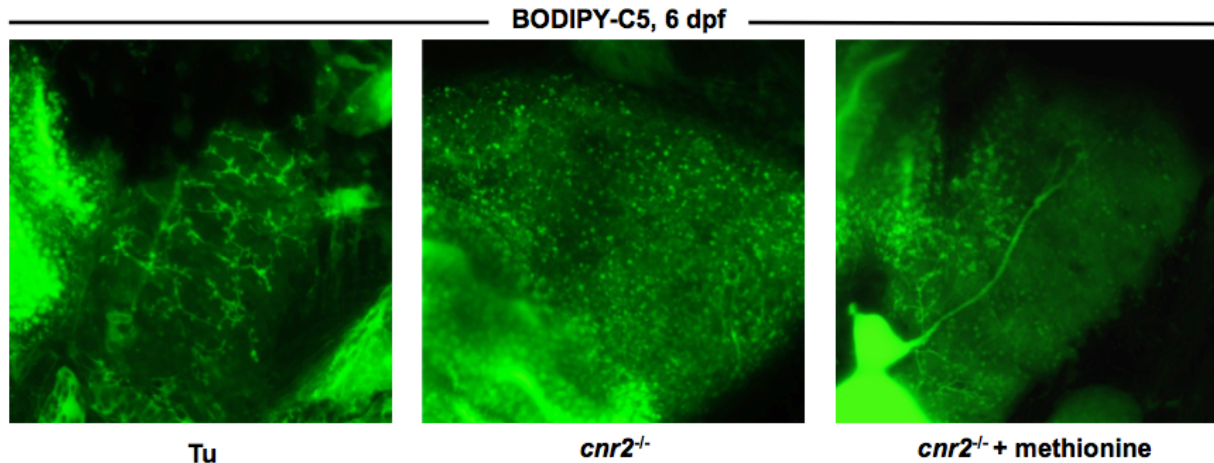


Figure S2-12. Methionine treatment partially rescues the lipid processing defect in *cnr2*^{-/-} mutants

To determine if methionine treatment impacts the lipid deposition and decreased biliary branching seen in *cnr2*^{-/-} mutants, we treated *cnr2*^{-/-} mutants with methionine beginning at 24 hpf, followed by administration of BODIPY-C5 fluorophore at 6 dpf. We imaged the digestion of the C5 fatty acid molecule by confocal microscopy and observed that while there was extensive branching observed in wild type larvae, there was decreased branching with lipid and cholestatic deposition in the *cnr2*^{-/-} mutants. However, the *cnr2*^{-/-} mutants treated with methionine showed increased biliary branching and decreased fatty accumulation. These images suggest that methionine treatment beginning in early embryonic development may prevent the fatty phenotype observed in 6 dpf *cnr2*^{-/-} mutants. Representative images are shown.

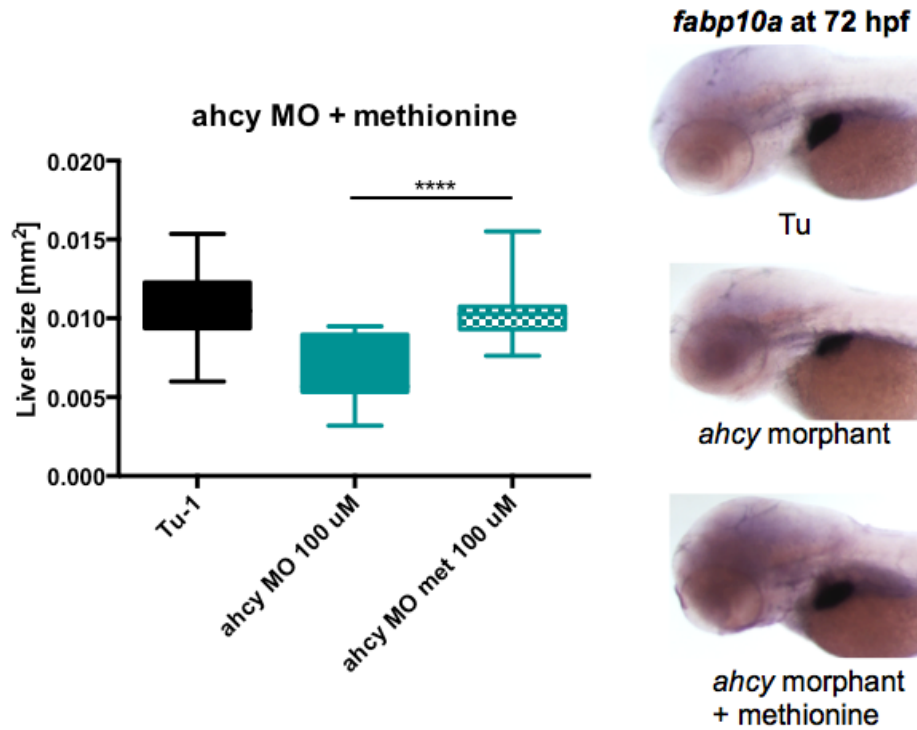


Figure S2-13. Methionine rescues liver development defect in *ahcy* morphants

As a positive control, we assessed the role of methionine treatment in the context of a known methionine metabolism disruption. Adenosylhomocysteine hydrolase (*ahcy*) is an essential enzyme in this pathway and functions to break down S-adenosylhomocysteine into adenosine and homocysteine. Mutants of this gene display hepatic steatosis and liver degeneration, followed by larval lethality (Matthews et al., 2009). Morpholino knockdown of *ahcy* resulted in smaller liver size at 72 hpf; however, treatment with methionine from 24-72 hpf rescued this liver size defect. This rescue indicates that methionine metabolism has a crucial role in the determination of liver size during hepatogenesis.

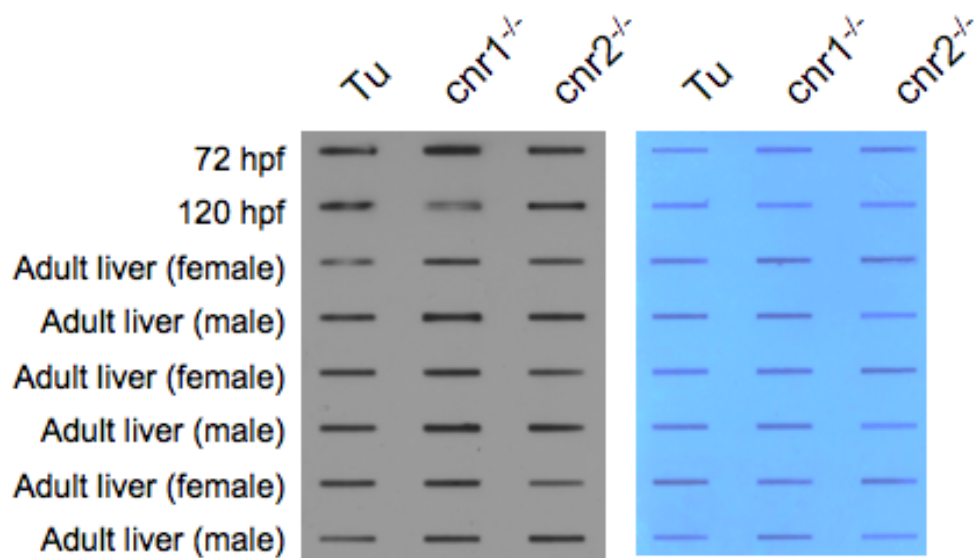


Figure S2-14. DNA methylation in cannabinoid receptor mutants

We examined global DNA methylation levels by DNA slot blot. We extracted DNA from 72 hpf or 120 hpf *cnr1*^{-/-} and *cnr2*^{-/-} whole larvae or adult liver samples, and blotted for 5-methylcytosine in samples with standardized DNA concentrations. This figure shows DNA slot blot of 5-methylcytosine expression in DNA samples from 72 hpf and 120 hpf whole zebrafish larvae, or adult zebrafish livers. There are no consistent differences in 5-methylcytosine expression in *cnr1*^{-/-} and *cnr2*^{-/-} mutant samples compared to controls. Methylene blue staining is shown at right as a loading control. Unlike western blotting, which separates a mixture of proteins on a gel and allows for visualization of multiple proteins that may be differentially affected by decreased methylation, the DNA slot blot technique may not be sensitive enough to distinguish among DNA methylation changes in specific genomic regions.

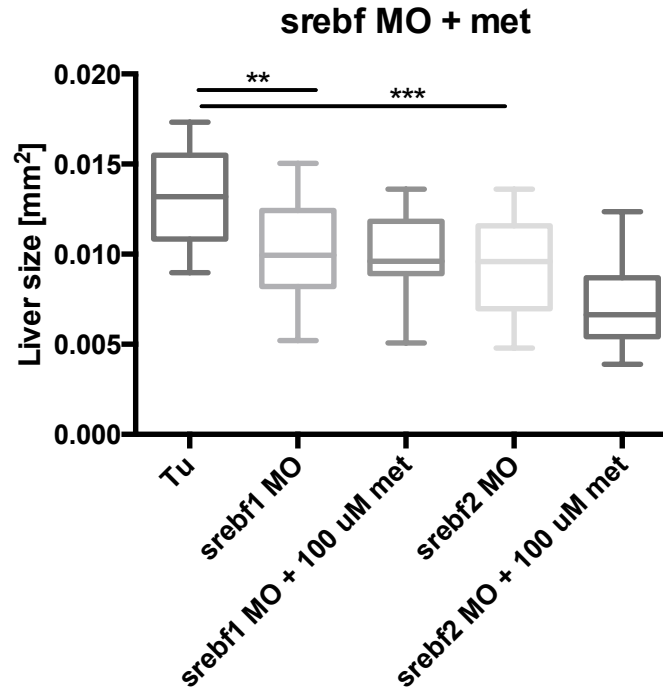


Figure S2-15. Methionine treatment does not rescue liver size in *srebf1* and *srebf2* morphants

To establish an epistatic relationship between methionine metabolism and *srebf1* and/or *srebf2*, we knocked down *srebf1* and *srebf2* using morpholinos and treated morphant embryos with methionine. Morphants exhibited smaller livers at 72 hpf, but methionine failed to impact liver size. Increasing methionine concentration up to 500 μ M had no effect (data not shown). In this setting, transient knockdown using morpholino may not have been sufficient for studying the impact of methionine treatment or, alternatively, *srebf1* and *srebf2* may link to methionine metabolism via a feedback loop that cannot be rescued.

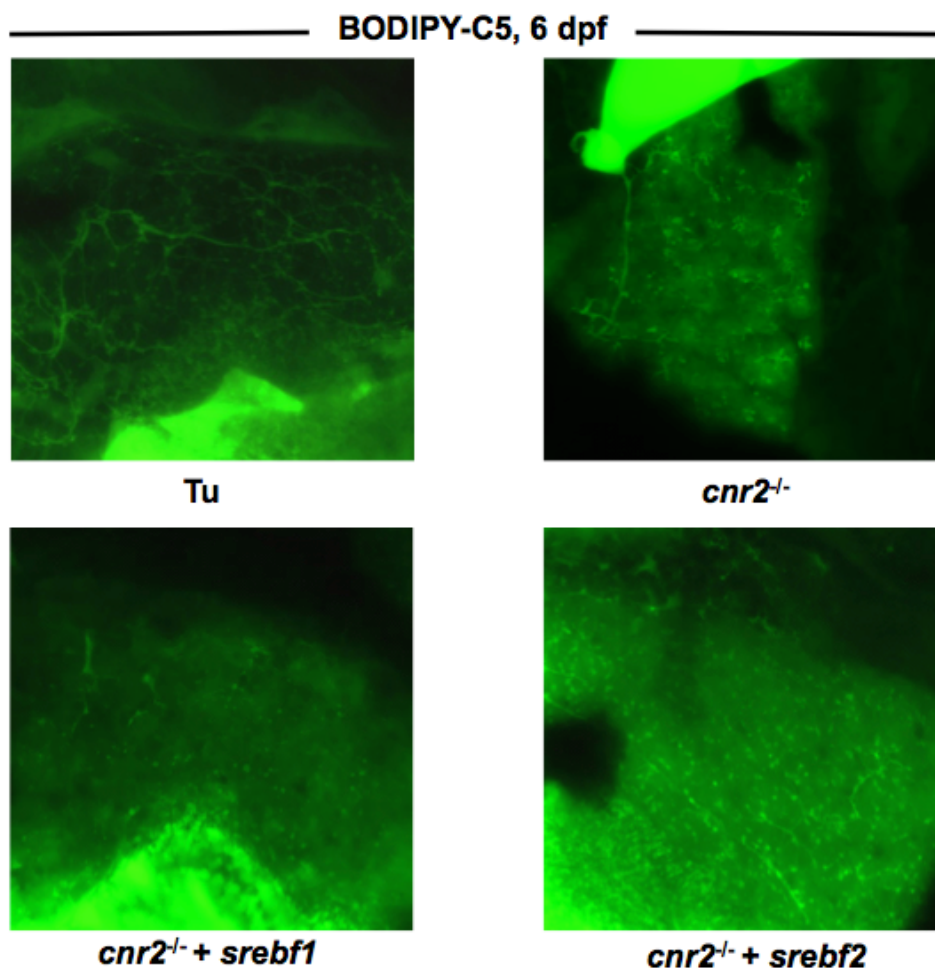


Figure S2-16. SREBP overexpression does not rescue lipid processing defects

To determine if overexpression of *srebf1* or *srebf2* mRNA can rescue the aberrant lipid processing phenotypes observed in *cnr2^{-/-}* larvae, we administered BODIPY-C5 fluorophore at 6 dpf to *cnr2^{-/-}* mutants alone or with *srebf1* or *srebf2* mRNA injection. We imaged liver morphology by confocal microscopy and did not observe major differences between *cnr2^{-/-}* mutants and larvae with mRNA overexpression. One explanation for lack of rescue could be that mRNA overexpression at the one-cell stage is only transient and becomes diluted by 6 dpf. Alternatively, the defects caused by aberrant signaling through the CB2 receptor may be too severe for rescue by overexpression of a downstream regulator and an alternate pathway may be responsible for the observed effects.

Appendix 3: Chapter 3 Supplemental Data

Table 3-2: ATG-site morpholinos designed to candidate genes

Gene	Target Sequence
<i>pnpla3</i>	5' TCCAGCCTTCCTTCAAATCAAACAT 3'
<i>cpn1</i>	5' AGATGAGAGAGCTGCCTGACAGCAT 3'
<i>trib1</i>	5' GGTTATGAATCCACTGCACGCTCAT 3'
<i>fads2</i>	5' TCCGCCACCCATCGCTGATCTCTGA 3'
<i>mif</i>	5' TTGTGTTCACTACAAACATCGGCAT 3'
<i>slc2a2</i>	5' ACTGCTTCTCCATTTTGCATGAAGT 3'
<i>pklr</i>	5' AGTAACGCCGGATACGAGCACCCAT 3'
<i>mapk10</i>	5' ACGTCTGTTCATAAATACCTTGCAT 3'
<i>aldob</i>	5' AGCAGCACACGGGATTAAAAGTGTA 3'
<i>efna1a</i>	5' TGTATAAAAGCCACACCAGATCCAT 3'
<i>efna1b</i>	5' CGCACAGCAGCCACAGAAAATCCAT 3'
<i>samm50</i>	5' GTGTACGGTCCCCATGTTTAAAAGA 3'
<i>mical3a</i>	5' CCGCATTGACGCCTCCATCTCCCAT 3'
<i>mical3b</i>	5' TCTCAGACTGACCGTCCCACATCGC 3'

Table 3-3: Splice-site morpholinos designed to candidate genes

Gene	Target Sequence
<i>pnpla3</i>	5' ATACATACAGTGGCATTACCTATGC 3'
<i>cpn1</i>	5' CTCCACTGAAAAACACAGGTCAGGT 3'
<i>trib1</i>	5' TATAGTATGCAGCGGTTTACCTTGC 3'
<i>fads2</i>	5' CCTTTAATATTTGACTCACCGTCTC 3'
<i>mif</i>	5' ATGTACTGTCACACAGACAAACACA 3'
<i>slc2a2</i>	5' AACTGCTGCCAGACATGAAAACACA 3'
<i>pklr</i>	5' GATACGAGCTGAATGATACACACAT 3'
<i>mapk10</i>	5' ATCCACTGCAGGCAAAGAAGCATGA 3'
<i>aldob</i>	5' GTGCCTAGAACATAAAACGGTCACA 3'
<i>efna1a</i>	5' AAGTGCAACTCACAGATGTAGTAGT 3'
<i>efna1b</i>	5' AGGCAAATTATGGACACTTACAGAT 3'
<i>samm50</i>	5' TGACATTATGTCCATCTTACATCTT 3'
<i>mical3a</i>	5' CCCAGGGAAACTTTCATACCTTAGT 3'
<i>mical3b</i>	5' ACAACACAAGTGATACTTACCGTGC 3'

Table S3-1: PCR primers for sequencing of ATG-site morpholino target region

Gene	Target Sequence
<i>pnpla3</i>	5' CCGCAGCTGTATCCCTTCTA 3', 5' GCATTACCTATGCAAGCCTCT 3'
<i>cpn1</i>	5' CAGCAATCATGCCTCAAAAA 3', 5' GCCCTCACCATCTCCTCATA 3'
<i>trib1</i>	5' TTGCGTGAAATCAGACGGTA 3', 5' ACTCTCAGCCAGGAAGGACA 3'
<i>fads2</i>	5' TTTGCTTTTGTGACCCAGTG 3', 5' GCACCAAGCCAATGTTTCTA 3'
<i>mif</i>	5' GCGGGGTTTTACGGTAGTCT 3', 5' GTGCTTACAGTTTTCTATCCCATCT 3'
<i>slc2a2</i>	5' AAGGTCTGATCCCAGCACAC 3', 5' CTCGGTCAATTTTGCAGTT 3'
<i>pklr</i>	5' AGCTTCAGGGAAGATGAGCA 3', 5' TTTGGGAGAGGGATGATTTG 3' and 5' TGCCTGAAGTCTTTTCTCTTATTTT 3', 5' TCCAGCAGACACAGATGCTC 3'
<i>mapk10</i>	5' TGCCTTGTTGAGCGTAGTTG 3', 5' CTAAGGAGGGGTTTGGAAC 3' and 5' CCTTCACGAACACCATCAGA 3', 5' CTCACAGAATGGAAGTGAATGAA 3'
<i>aldob</i>	5' ATCGTTCAGTCCCGATTACAC 3', 5' CACTTGCATGTTACGGTTC 3'
<i>efna1a</i>	5' CGCGGATCTCTTGATTCACT 3', 5' CGAACACAGCGCAGATACTC 3'
<i>efna1b</i>	5' GCATTTAACCGCGTCTGTTT 3', 5' CGCACACAGCTTGCATTAAG 3'
<i>samm50</i>	5' CAACGTGACGTGGTTGTTCT 3', 5' GTTTCTGAACTCGGCCTGTC 3'
<i>mical3a</i>	5' TCATTCAGGGTGAAAATGTCC 3', 5' AAGCCTTGAGAGTGCCTTTG 3'
<i>mical3b</i>	5' ATTGTCTGCTCCCCATTACAG 3', 5' TTTCTGGCAGGCTCTTTTGT 3'

Table S3-2: Effect of gene knockdown using splice site morpholinos on hepatic progenitor population (*hhex* expression).

Morpholino	% small	% normal	% large	n	Significance
control	21.2%	73.7%	5.1%	99	
<i>pnpla3</i>	43.8%	56.3%	0.0%	112	p < 0.05
control	18.1%	71.4%	10.5%	105	
<i>cpnl</i>	19.0%	71.4%	9.5%	84	n.s.
control	14.3%	80.0%	5.7%	35	
<i>trib</i>	25.0%	65.0%	10.0%	40	n.s.
control	10.2%	81.6%	8.2%	49	
<i>fads2</i>	16.0%	76.0%	8.0%	50	n.s.
control	21.2%	61.5%	17.3%	52	
<i>mif</i>	24.1%	70.4%	5.6%	54	n.s.
control	16.7%	72.9%	10.4%	48	.
<i>slc2a3</i>	15.1%	77.4%	7.5%	53	n.s
control	24.6%	69.2%	6.2%	65	
<i>pklr</i>	43.4%	55.8%	0.9%	113	p < 0.05
control	12.3%	80.7%	7.0%	57	
<i>mapk10</i>	34.7%	61.3%	4.0%	75	p < 0.05
control	19.2%	73.1%	7.7%	78	
<i>aldob</i>	29.5%	65.3%	5.3%	95	n.s.
control	38.1%	61.9%	0.0%	21	
<i>efna1a</i>	48.1%	51.9%	0.0%	27	n.s.
control	23.9%	71.7%	4.3%	46	
<i>efna1b</i>	32.1%	66.1%	1.8%	56	n.s.
control	15.2%	74.2%	10.6%	66	
<i>samm50</i>	15.9%	76.2%	7.9%	63	n.s.
control	18.5%	79.6%	1.9%	54	
<i>mical3a</i>	22.4%	74.1%	0.0%	58	n.s.
control	25.0%	67.6%	7.4%	68	
<i>mical3b</i>	25.3%	65.9%	8.8%	91	n.s.

Table S3-3: Effect of gene knockdown using splice site morpholinos on hepatocyte gene expression and liver size (*fabp10a* expression).

Morpholino	% small	% normal	% large	n	Significance
control	23.5%	70.6%	5.9%	34	
<i>pnpla3</i>	71.1%	26.3%	2.6%	38	p < 0.05
control	35.6%	53.3%	11.1%	45	
<i>cpn1</i>	78.7%	21.3%	0.0%	47	p < 0.05
control	36.4%	63.6%	0%	44	
<i>trib</i>	69.8%	30.2%	0%	53	p < 0.05
control	18.2%	74.0%	7.8%	77	
<i>fads2</i>	43.7%	53.5%	2.8%	71	p < 0.05
control	40.4%	51.1%	8.5%	47	
<i>mif</i>	31.0%	59.5%	9.5%	42	n.s.
control	23.0%	67.0%	10.0%	100	
<i>slc2a3</i>	54.7%	35.9%	9.4%	117	p < 0.05
control	26.2%	59.0%	14.8%	61	
<i>pklr</i>	67.2%	29.5%	3.3%	61	p < 0.05
control	26.9%	73.1%	0.0%	26	
<i>mapk10</i>	87.5%	12.5%	0.0%	48	p < 0.05
control	18.0%	72.1%	9.8%	61	
<i>aldob</i>	26.3%	63.2%	10.5%	57	n.s.
control	20.0%	75.0%	5.0%	40	
<i>efna1a</i>	26.8%	68.3%	4.9%	41	n.s.
control	30.4%	60.7%	8.9%	56	
<i>efna1b</i>	32.0%	60.0%	8.0%	50	n.s.
control	8.6%	80.0%	11.4%	35	
<i>samm50</i>	65.4%	34.6%	0.0%	26	p < 0.05
control	40.9%	54.5%	4.5%	44	
<i>mical3a</i>	42.9%	50.0%	7.1%	42	n.s.
control	24.4%	63.3%	12.2%	90	
<i>mical3b</i>	32.6%	61.1%	6.3%	95	n.s.

Table S3-4: Susceptibility to metabolic and toxic injury after gene knockdown using splice-site morpholinos

Gene knockdown	+2% EtOH (Oil Red O)	+2.5 mM APAP (<i>fabp10a</i> expression)
<i>pnpla3</i>	unchanged	reduced
<i>cpn1</i>	unchanged	unchanged
<i>trib1</i>	unchanged	reduced
<i>fads2</i>	unchanged	unchanged
<i>mif</i>	unchanged	unchanged
<i>slc2a2</i>	unchanged	unchanged
<i>pklr</i>	unchanged	unchanged
<i>mapk10</i>	unchanged	unchanged
<i>aldob</i>	unchanged	unchanged
<i>efna1a</i>	unchanged	unchanged
<i>efna1b</i>	unchanged	unchanged
<i>samm50</i>	unchanged	Trend toward reduced
<i>mical3a</i>	unchanged	unchanged
<i>mical3b</i>	unchanged	unchanged